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Donor MHC Class I Gene Transfer to Recipient Liver: mechanistic studies of a novel strategy for transplant tolerance induction

DR. DANIEL LEE JOHN BUNKER
DECLARATION

This thesis is the result of full-time study at the University of Sydney, Australia with the Collaborative Transplant Group between January 2013 and January 2014. All work, except where duly acknowledged, is my own. This work has not been submitted for another degree at this or any other university.

____________________

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BE (Aerospace) Hons I University Medal, MBBS Hons
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ABSTRACT

Transplantation remains life-saving treatment for patients with organ failure. Unfortunately, its benefits are often truncated by rejection due to activation of the recipient’s immune system to alloantigens primarily derived from donor MHC molecules. While we have made progress in the treatment of acute rejection, chronic rejection leading to progressive loss of allograft function remains the usual outcome. Furthermore, immunosuppressive therapy prescribed in the setting of transplantation has significant side effects including infection and malignancy resulting from a non-specific state of global immunosuppression, as well as toxicity to end organs. Interestingly, the liver has a unique propensity to confer immunological tolerance; liver transplants have been accepted across complete MHC mismatches and maintained without immunosuppressive therapy. Achieving this state of operational tolerance remains the goal for allotransplantation.

We have previously shown that transduction of recipient liver with donor MHC class I molecules (rAAVKn) using optimised recombinant adeno-associated viral vectors leads to acceptance of skin transplants in a single MHC class I mismatched murine model through ‘functional silencing’ of an expanded population of alloreactive CD8 T cells. Donor 178.3 strain mice expressing K\(^{k,b}\) were used as donors to B10.BR recipients expressing K\(^k\). We reconfirmed these findings, showing that operational tolerance harnessing the ‘liver tolerance effect’ is indeed achievable (grafts viable beyond 250 days).

To better understand the underlying immune mechanisms, we investigated the role of direct recognition of MHC class I by recipient T cells in tolerance induction. To do this,
we engineering an MHC class I molecule (rAAVD227K) with a single point mutation which abrogates CD8 co-receptor binding and therefore direct antigen recognition. In this system, skin grafts placed one week after inoculation with rAAVD227K are rejected at a reduced tempo compared to naïve B10.BR mice (MST = 27 days versus 16 days).

Examination of isolated hepatocytes shows transient upregulation of the programmed cell death ligand 1 at day 7 on animals given rAAVKb but not on those given rAAVD227K. PD-L1:PD-1 cross-talk between hepatocytes and CD8 T cells has been shown to cause inactivation and apoptosis of antigen-specific cytotoxic lymphocytes, which may underlie the induction of tolerance in rAAVKb–treated animals. Blockade of the PD-L1 ligand in Kb-transduced animals resulted in liver inflammation, but did not break tolerance to subsequent skin grafts. Concurrent administration of rAAVKb and anti-PDL1 resulted in inflammatory infiltrates and raised ALTs, perhaps due to activation of Kb-reactive clones normally silenced through PD-L1:PD-1 interactions. Administration of either rAAVKb or anti-PDL1 alone was not immunogenic. Naïve B10.BR mice primed by rejecting a 178.3 skin graft and then inoculated with rAAVKb a week after rejection displayed increased numbers of peribiliary FoxP3+ cells which presumably reflect a population of induced regulatory T cells. 

Taken together, we have shown that the ‘liver tolerance effect’ provides a powerful avenue for inducing tolerance even in primed recipients. Direct antigen recognition seems to play a pivotal role in inducing this state which is exemplified by increased numbers of regulatory T cells. Furthermore, it appears that PD-L1 is involved in, but is not a pre-requisite for, developing tolerance to alloantigens and subsequent allograft acceptance.
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ACRONYMS AND ABBREVIATIONS

AAV – adeno-associated virus

AICD – activation-induced cell death

AIRE – autoimmune regulator

ALT – alanine aminotransferase

AMR – antibody-mediated rejection

AP-1 – activator protein 1

ApoE – Apolipoprotein E

APC – antigen presenting cell

ATRA – all-trans retinoic acid

BAFF – B-cell activating factor

BMT – bone marrow transplantation

BSS – balanced salt solution

CAT – composite tissue allotransplantation

CDR – complimentary determining region

CMV – choriomeningitis virus

DAMPS – damage associated molecular patterns
DC – dendritic cell

DNA – deoxyribonucleic acid

DSA – donor-specific antibody

dsDNA – double-stranded deoxyribonucleic acid

EDTA – ethylenediaminetetraacetic acid

ELISPOT – enzyme-linked immunosorbent spot

FGL2 – fibrinogen-like protein-2

FoxP3 – forkhead box P3

GATA-3 – GATA-binding protein 3

hAAT – human α-1 antitrypsin

HLA – Human Leukocyte Antigens

HSC – hepatic stellate cell (Ito cell)

ICOS – Inducible T cell costimulator

IDO – indoleamine 2,3-dioxygenase

IFN – interferon

IRI – ischaemia reperfusion injury

ITR – inverted terminal repeat

IVlg – intravenous immunoglobulin
KC – Kupffer cell

KO – knock out

LAG-3 – lymphocyte-activation gene 3

LSEC – liver sinusoidal endothelial cell

MAP – mitogen-activated protein

MCS – multiple cloning site

MHC – major histocompatibility complex

MICA – MHC class I-related chain A

miH – minor histocompatibility antigen

MST – median survival time

NFAT – nuclear factor of active T cells

NF-κB – nuclear factor kappa-light-chain-enhancer of activated B cells

NHS – normal horse serum

NK – natural killer (cell)

Nrp-1 – neuropilin-1

ORF – open reading frame

PBS – phosphate buffered saline

PD-1 – programmed cell death protein 1
PD-L1 – programmed cell death ligand 1

PK – protein kinase

RAG – recombinase activating gene

RBE – Rep Binding Element

RORγt – RAR-related orphan receptor gamma

SMAC – supramolecular activation cluster

SMAD – mother against decapentaplegic

ssDNA – single-stranded deoxyribonucleic acid

STAT – signal transducers and activators of transcription

SURE2 – stop unwanted rearrangement events 2

TCR – T cell receptor

TDW – triple distilled water

TIM-3 – T cell immunoglobulin- and mucin-domain-containing molecule

TNF – tumour necrosis factor

TRA – tissue restricted antigen

WPRE – woodchuck hepatitis virus posttranscriptional regulatory element)

WT – wild type

ZAP70 – zeta-chain associated protein kinase 70
**Units**

cc – cubic centimeter

kDa – kilodaltons

L - litre

ml – millilitre

mm – millimetre

rpm – revolutions per minute

μg – microgram

U/L – units per litre

vgc – vector genome copies
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**DEFINITIONS**

**Autograft** – graft from one part of the body to another on the same individual

Isograft (syngeneic graft) – graft between genetically identical individuals

**Allograft** – graft between two genetically distinct individuals of the same species

**Xenograft** – graft between individuals of a different species

**Alloantigens** – molecules which are recognised as foreign on allografts

**Alloreactive** – describing components of the immune system that react to an allograft

**Allorecognition** – recognition of antigen of ‘non-self’ origin

**Alloresponse** – the effector mechanism directed against an allograft

**Rejection** – the immune reaction of a recipient against foreign donor tissue

**Self-tolerance** – the lack of an immune response to self-antigens

**Tolerance** – unresponsiveness to an antigen that would usually be immunogenic

**Operational tolerance** – antigen-specific unresponsiveness in the absence of immunosuppression
1.0 INTRODUCTION

‘Man has, as it were, become a kind of prosthetic God. When he puts on all his auxiliary organs, he is truly magnificent; but those organs have not grown on him and they still give him much trouble at times’ - Sigmund Freud

1.1 CURRENT STATUS OF CLINICAL ALLOTRANSPLANTATION

Organ transplantation remains life-saving treatment for patients with end-organ failure due to congenital or acquired disease [1-3]. We are also seeing a rise in the rate of composite tissue allografts for return of form and function procedures [4]. Transplant medicine has evolved into a specialised field targeting the functional replacement of a range of solid organs. Transplants of the kidneys, heart and lungs are performed most commonly. Pancreas transplant is sometimes performed for patients with type 1 diabetes, often in concert with renal transplant for concurrent end-stage kidney disease. Less commonly, the thymus can be transplanted in patients with absent or hypoplastic thymus tissue, while intestinal transplant is sometimes employed in patients with irreversible intestinal failure who develop life-threatening complications from total parenteral nutrition. For patients with end stage heart, lung or liver failure, transplant remains the only life-saving treatment option. Concurrently, there is a global epidemic of non-communicable diseases such as diabetes and obesity, resulting in high rates of end-organ damage and further increasing the need for solid organ transplantation [5-7]. While the advent of more powerful and newer immunosuppressive agents has reduced rates of acute organ rejection, long-term
allograft survival remains poor [8-11]. As physicians we therefore face two acute issues: meeting the demand for transplantable organs and maximising graft survival.

The demand for organ transplantation is immediate and increasing. Since 1993, there have been a total of 19,034 renal transplants in Australia, with 8,382 grafts still functioning at the end of 2010 [2]. In 2010, 846 kidney transplants were performed in Australia alone, with 35% of these being from living donors and 88% of all recipients receiving their first organ [2]. Only 7% of patients on dialysis for end stage renal disease in Australia received a transplant in 2010, the remaining patients remaining on haemo- or peritoneal dialysis: at end of 2011, there were still 1,135 patients awaiting renal transplant [2].

Episodes of rejection of primary renal grafts from both living and deceased donors six months after transplant are 16% and 16.4% respectively, with no improvement for patients receiving retransplantation [2]. With modern therapy, 95% of primary deceased donor grafts are still functioning one year post transplant, with 65.7% of those transplanted in 2000-2004 still functioning ten years later: for re-grafts the rates are 91% and 48.5%, respectively [2]. There has been only a marginal increase in long-term renal transplant survival (approximately 2 years) between 1989 and 1995, mainly driven by increased survival in re-transplanted patients [10]. A critical appraisal of 252,910 single organ renal transplants (deceased and living donor) undertaken between 1989 and 2009 in the United States confirms only marginal improvements in long-term survival: half-lives for deceased-donor transplants were 6.6 years in 1989, 8 years in 1995 and 8.8 years in 2005 [9]. The main improvements were seen in short-term survival (prevention of acute rejection) and in high-risk subgroups such as recipients of
grafts from expanded criteria donors. Data from Australian registries supports these findings, with the most significant cause of graft loss now chronic allograft nephropathy. We are not meeting the demand for transplantable organs and long-term renal graft survival is inadequate. Moreover, mortality in renal transplant patients remains higher than for age-matched controls [12].

For type 1 diabetic patients with end stage renal failure, there were 26 combined kidney-pancreas transplants performed in Australia in 2011, as well as 9 pancreas islet cell transplants [3]. At that time there were 49 people awaiting kidney-pancreas transplant and 7 people awaiting pancreatic islet cell transplant [3]. The common theme remains a demand which we are not able to reconcile with current organ availability.

The latest data from the Australia and New Zealand Cardiothoracic Organ Transplant Registry shows that the rate of cardiac transplants has remained relatively stable over the last decade, with 76 performed in Australia and New Zealand in 2011 [1]. Additionally, there were 2 heart-lung, 12 single lung and 158 double lung transplants [1]. As of January 2011, there were 53 people awaiting cardiac transplant and 124 people awaiting lung transplant, with a mean waiting time of 171 and 197 days, respectively [1]. Between 1984 – 2011, 10% of heart transplant patients died from acute rejection: lung transplant patients fared better from a rejection point of view, with 3% of deaths attributable to acute rejection between 1986 and 2011 [1]. Unfortunately, bronchiolitis obliterans syndrome remains a major cause of graft loss in the years following lung transplant. Again, improvements in both organ supply and graft survival are necessary.
Concurrently, we are seeing increasing rates of vascularised composite allotransplantation (CAT) procedures such as hand and face transplant utilised for return of form and function. The first face transplant was performed in France in 2005 with more than 15 full or partial face transplants completed subsequently [13]. Hand transplant was trialled much earlier in Ecuador in 1964 but complicated by acute rejection [14, 15]. The first successful hand transplant was performed in 1998 by Jean-Michel Dubernard and since then over 60 hand transplants have been performed worldwide [16]. Between 1998 and 2010, 49 hand transplants were performed and 85% of these have had at least one episode of acute rejection despite immunosuppression [4]. In Europe and the United States of America, graft survival remains over 90% for hand transplant [17]. While outcomes look promising, powerful life-long immunosuppressive regimes after CAT are not without risk [18].

Organ transplantation between genetically distinct individuals usually requires ongoing immunosuppressive therapy. Such agents are administered life-long and associated with significant side effects such as nephrotoxicity, dyslipidaemia and diabetes [19, 20]. Their lack of specificity leads to a global state of reduced immunocompetence for the host, thereby increasing the risks of cancer and infection [21, 22]. Immunosuppressive agents mainly target the activation or proliferation of T cells which are critical to the process of transplant rejection, while monoclonal antibodies act to deplete B or T cell populations [23]. Regimes usually consist of induction (depletion) and maintenance therapy, with additional treatment administered for episodes of acute rejection. Commonly used immunosuppressive agents include cyclosporine, tacrolimus, mycophenolate mofetil and prednisone.
Episodes of acute rejection may be attenuated with antibody therapy (such as the anti-T cell antibody Muromonab-CD3) and/or increased doses of maintenance agents.

To extend transplant half-lives and avoid the toxicities and side-effects of lifelong immunosuppressive therapy, the focus shifts to modulating an individual’s immune response to transplanted tissues so that they are accepted as ‘self’. This is the concept of transplant tolerance. To begin to understand how this can be achieved, we will first examine the way in which the immune system discriminates self and non-self, the immune response to foreign tissues, alloreognition, alloresponse (rejection) and finally some of the progress made so far in achieving tolerance. Rejection, the immune response to organ allografts, remains the ultimate barrier to successful long-term graft survival. The principal antigens which elicit this immune response are encoded within the Major Histocompatibility Complex (MHC). MHC molecules are central to alloreognition, antigen display and T cell activation.

1.2 THE MAJOR HISTOCOMPATIBILITY COMPLEX

‘The unique footprint on every nucleated cell within an individual which identifies it as ‘self’ is the Major Histocompatibility Complex. MHC molecules display antigens to cells of the immune system and help to activate such cells if that antigen is recognised as ‘non-self’ (foreign)’ – Anon

1.2.1 HISTORY

MHC genes (tissue antigens) was discovered during research into tumours and tissue transplant rejection in murine models. In the early 1900’s, Loeb and Tyzzer postulated
that rejection may have a hereditary (i.e. genetic) basis [24, 25]. Even as far back as this, patterns of rejection between transplanted tissues were well-recognised. George Schone had collated work on tissue transplants as far back as 1912 and postulated six laws of transplantation which are still accurate today: (1) xenografts universally fail, (2) allografts usually fail, (3) autografts generally succeed, (4) allografts experience rejection after an initial take, (5) secondary allografting between the same pair of animals leads to more rapid rejection, and (6) the closer the ‘blood relationship’ between donor and recipient, that greater the chance that the graft will take [26]. Remarkably, such accurate observations had been made prior to any understanding of the role of genetics or the immune system’s response to transplantation.

Little postulated that a genetic basis for rejection may be inherited in a Mendelian pattern [27]. In 1916, Little and Tyzzer performed extensive studies of tumour transplant acceptance between different hybrid strains of mice in an attempt to identify modes of inheritance of susceptibility [28, 29]. Subsequently, Bover made the important discovery that transplants were accepted if the donor and recipient were monozygotic twins [30]. Research by Little, Bittner, Cloudman and Strong continued in an attempt to identify the genetic basis of rejection, the findings of which were summarised by Bittner in 1935 and Little in 1941 [31, 32]. It was Haldane who postulated that rejection was controlled by an immune response against transplanted cellular antigens which were structurally normal to the host, hence giving rise to the alloantigen hypothesis of tumour rejection [33]. Because tumour cells were not rejected when transplanted into an inbred strain, Haldane felt that the immune response was directed against alloantigens which were conserved even among neoplastic cells.
Sir Peter Medawar’s pioneering work using rabbit skin grafts in the 1940’s demonstrated that allografts were universally rejected in a predictable fashion. He found that rejection of a primary transplant, termed first-set rejection, usually occurred at a median of 10 days post-transplant: a second graft between the same donor and recipient elicited rapid graft failure at a median time of 6 days [34]. Medawar labelled this phenomenon second set rejection. Medawar’s pioneering work provided evidence that rejection was mediated by an actively acquired immune response, being preceded by a latent period prior to immune mediated destruction [34, 35]. These findings were later re-confirmed in studies by Billingham, Brent, Medawar and Sparrow [36]. The regional lymph nodes and spleen were found to confer the property of second-set rejection by transferring tissues from an actively immunised animal to mice which had not received a primary graft, providing evidence that allograft rejection was mediated by an adaptive immune response [37, 38]. Medawar’s work revealed that the immunological process of rejection was both dose-dependent and systemic in nature. He suggested that the intensity of the immune response was dependent on the genetic diversity between the donor and host and attempted to describe an antigenic model for skin transplant rejection, thus ascribing an immunological hypothesis for organ rejection. The ability of a graft to be accepted by a recipient is determined by the degree of histocompatibility between the donor and recipient animal.

The gene cluster controlling rejection was first identified by Gorer in 1937 when he found an association between tumour rejection and the expression of certain blood group antigens [39]. In his experiments, Gorer discovered that one of these antigens, which he labelled antigen 2, conferred rejection: a mouse without antigen 2
expression rejected transplants from a mouse expressing the blood group antigen 2. The gene responsible for encoding antigen 2 segregated with the genes responsible for rejection, and serum from mice that rejected tumour cells showed antibodies to this antigen. Meanwhile, Snell bred strains of mice that differed only at the locus controlling rejection: he termed this the ‘H’ or histocompatibility locus [40]. The terms were subsequently joined and the mouse histocompatibility complex was then denoted H2. The observation that hybrid strains of mice (A x B) universally accept allografts from either A or B inbred strains while an A or B strain animal will reject a graft from the hybrid led to the conclusion that H2 alleles are polymorphic and codominantly expressed.

It wasn’t until the 1950’s that typing of the human MHC complex began, now referred to as the Human Leukocyte Antigen (HLA) complex as a result of its discovery on leukocytes. The first HLA antigen (HLA-A2) was discovered by Dausset in 1958 while investigating the Hu-1 locus in man, a region which was found to control mixed leukocyte reactions and specificities to cytotoxic antisera, both of which were known to have predictive value in skin transplant survival [41, 42]. 1962 saw Jan van Rood and colleagues identify the 4A and 4B series (now termed HLA-Bw4 and HLA-Bw6), followed by the discovery of the LA series (HLA-A1, HLA-A2, HLA-A3) by Payne and Bodmer in 1964 [43, 44]. Bernard Amos, a colleague of Gorer, subsequently organised the first International Histocompatibility Working Group in 1964 and the first World Health Organisation Nomenclature Committee Meeting in 1968, which have served to collate and standardise subsequent typing of MHC alleles through to the modern era.
Despite awareness of the association between MHC genes and susceptibility to disease and rejection, their function in healthy individuals was not appreciated until some time later. Benacerraf and McDevitt explored inbred strains of animals and their ability to form antibodies to simple antigenic peptides: they discovered that the MHC genes controlled the ability to mount an immune response and determined interactions between lymphocytes [45-47]. The genes responsible for conferring this ability were named **immune response** (Ir) genes and are situated in the MHC class II region. It became evident that the major histocompatibility complex was important in determining not only rejection of transplanted tissue, but was also critical for mounting an immune response to pathogens. The mechanisms underlying this interplay of MHC with lymphocytes was brought to light by the discovery of MHC restriction by Zinkernagel and Doherty (see *The Major Histocompatibility Complex: Synthesis and Function*) [48, 49].

1.2.2 **Murine MHC (H2) and Human MHC (HLA)**

The MHC gene complex contains three regions denoted class I, class II and class III. There is striking similarity between murine (H2) and human (HLA) MHC gene complexes in terms of expression, structure and function. Like HLA, H2 comprises class I and class II genes which code for proteins involved in antigen processing as well as a class III region which produces cytokines and inflammatory mediators. Mouse MHC occupies approximately 2,000 kilobases and the arrangement of loci differs from that of humans [50]. Mice have three class I (K, D, L), two class II (I-A, I-E) and a range of class III genes located on chromosome 17 (see **Table 1** based on [51]) [52, 53].
The HLA complex is located on chromosome 6p21.31 and occupies approximately 0.1% of the human genome at 400 million base pairs: these are the most highly polymorphic loci known [53]. By 2010, there were already 2,558 class I and class II alleles recognised [54]. Codominant inheritance confers expression of both inherited alleles at class I (A, B, C) and class II loci (DP, DQ, DR) [55]. Class I genes encode three families of peptide binding proteins (‘major genes’ A, B and C): the major genes encode the α-chain which may be of subtype A, B, or C and are sometimes referred to as la genes [56]. The genetic locus for class II is denoted class D and contains families P, Q and R involved in peptide binding and other families (O and M) associated with processing antigens for display. The class II regions also encodes the immunoproteasome which generates peptides from ubiquinated cytosolic proteins for display on MHC class I molecules [57]. Genes are denoted by class, family and chain:
hence HLA-DQA corresponds to the α-chain of the Q family in class D. Mouse MHC class I H2-K, H2-D and H2-L genes are the correlate of human HLA-A, HLA-B and HLA-C genes, whereas the MHC class II genes I-A and I-E (Ir genes) are analogous to human HLA-DP, HLA-DQ and HLA-DR.

Transgenic strains of mice differing at a single MHC locus provide a convenient experimental platform to investigate immunological phenomena such as rejection and tolerance. The haplotypes of the murine strains used in this thesis are given in Table 2.

<table>
<thead>
<tr>
<th>H2 Haplotype</th>
<th>Class I</th>
<th>Class II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>K</td>
<td>D</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>B10.BR</td>
<td>k</td>
<td>k</td>
</tr>
<tr>
<td>178.3</td>
<td>k,b</td>
<td>k</td>
</tr>
</tbody>
</table>

Table 2: Mouse strains used in this thesis

1.2.3 General Structure

MHC molecules are members of the immunoglobulin superfamily. Structurally, they are composed of a variable extracellular domain, an invariant extracellular invariant immunoglobulin-like domain, a transmembrane portion which anchors the MHC into the cell membrane and a cytoplasmic region which plays a role in signal transduction. The variable portions of the MHC molecule include the peptide binding groove (or ‘cleft’) which presents antigen and adjacent amino acid sequences which are recognised by hypervariable complementarity determining regions (CDRs) of the T cell
receptor (CDR1 and CDR2). This cleft is formed by paired α helices resting on a β-pleated sheet [58]. Antigens are presented by either class I or class II MHC molecules, which differ in their structure and ability to accommodate antigenic peptides.

1.2.4 Class I

All nucleated cells express MHC class I molecules. Class I MHC molecules are composed of an α-chain (the ‘heavy chain’) non-covalently bound to β2 microglobulin (the ‘light chain’) (see Figure 1) [56]. The α-chain is comprised of five parts: subunits α1, 2, and 3, a transmembrane region which anchors the MHC complex into the cell wall and a carboxyl-terminus which lies in the cytosol [56]. The amino-terminal ends of the α1 and 2 proteins form the peptide binding cleft for antigen, whereas the α3 component is associated with the β2 microglobulin protein [59]. The peptide binding cleft contains a groove which anchors the antigen (8-11 amino acid peptides) and is flanked by framework determinants which contact the T cell receptor (CDR1, CDR2). Because only small peptides can be accommodated in the class I MHC groove, antigens must be processed prior to display. The α3 immunoglobulin-like portion is invariable and provides the binding site for the CD8 T cell co-receptor: this binding site is a negatively charged acidic loop (residues 222-229) which must be conserved to confer direct antigen recognition by CD8 T cells [60, 61]. MHC class I molecules are thus involved in stimulating CD8 cytotoxic T lymphocytes. The complete class I MHC is a heterotrimer and requires amalgamation of the heavy chain, light chain and peptide to confer molecular stability and subsequent expression on the cell surface [59].
1.2.5 Class II

Professional antigen presenting cells (B cells, Langerhans cells, monocytes, and macrophages) as well as activated T cells and endothelial cells express MHC class II molecules [62]. Class II MHC molecules are composed of non-covalently bound polymorphic α and β polypeptides which each contain four regions (see Figure 2) [63]. Both α1 and β1 associate to form the peptide binding groove, which can display much larger peptides than MHC class I (over 30 amino acids) [59]. The proximal ends of both proteins are invariable and are composed of an immunoglobulin-like region (α2 and β2), a hydrophobic transmembrane region for anchoring the MHC molecule and a cytosolic carboxyl-terminus end [56]. Usually both α and β proteins are encoded by the same class II MHC locus [59]. Of note, the β2 region exhibits a binding site for the T
cell co-receptor CD4. As such, MHC class II molecules are involved in stimulating CD4 (helper) T cell responses. The stably expressed MHC Class II molecule is again a heterotrimer. Class II genes also encode HLA-DM or H2-DM, an important protein involved in antigen processing and allore cognition via the indirect pathway (see The Major Histocompatibility Complex: Synthesis and Function).

![Structure of the MHC class II molecule](image)

*Figure 2: Structure of the MHC class II molecule*

(Adapted from Abbas, Lichtman & Pillai, 2012)

1.2.6 **CLASS III**

The class III MHC region contains genes which encode a number of proteins including those of the complement system (C2, C4a, C4b, factor B), enzymes related to lipid and steroid metabolism, inflammation (RAGE), cytokines, tumour necrosis factor and heat shock proteins [64-66].
1.2.7 SYNTHESIS AND FUNCTION

Early studies into tumour and skin transplants revealed that the phenomenon of graft rejection was immune-mediated. Additional work identified the genetic region responsible for controlling rejection as the major histocompatibility complex, and investigators such as Benacerraf and McDevitt found that this complex also controlled an individual’s ability to mount an immune response to different pathogens. It became clear that the MHC played a pivotal role in the normal immune response. The groundbreaking work of Zinkernagel and Doherty helped to characterise the importance of MHC in its interaction with T cells [48, 49]. These Nobel laureates discovered that T cells are MHC restricted: that is, T cells are only able to recognise antigen when associated with MHC molecules, from which it follows that MHC is of crucial importance in the adaptive immune response.

All nucleated cells express class I MHC molecules which identify it as ‘self’. Conversely, class II molecules are normally present only on cells which play a role in antigen presentation, B cells and activated T cells. Class I MHC molecules display cytosolic peptides such as those derived from intracellular pathogens and present antigen to cytotoxic (CD8) lymphocytes. Professional antigen presenting cells sample pathogens from the extracellular milieu, break them down in lysosomes and incorporate the resulting antigenic peptides with newly formed class II molecules which are then displayed on the cell’s surface. It is conventional to ascribe the presentation of endogenous antigens to class I MHC and the presentation of exogenous antigen to class II MHC: ‘cross-presentation’ (see below) is the exception to this rule [67].
Class I MHC molecules are synthesised in the endoplasmic reticulum of nucleated cells but require association with antigen prior to expression. Intracellular antigens (pathogenic or self) are ubiquinated and degraded by proteasomes before being transported to the endoplasmic reticulum by TAP (transporter associated with antigen processing) proteins [68]. TAP is located on the membrane of the endoplasmic reticulum and associates with tapasin which resides on the luminal side: it has a discrete affinity for newly formed MHC class I molecule and a chaperone protein termed calreticulin [69]. Together, these proteins ensure that MHC class I molecules are brought into contact with intracellular peptide antigens: subsequent binding of a peptide into the MHC groove confers stability to the complex with consequent transport to the cell surface. This pathway of antigen presentation is termed the endogenous pathway [70]. As intracellular pathogens are eradicated by cell-dependent killing, the association of MHC class I with CD8 T cells is elegant.

MHC class II molecules are associated with CD4 T cells which stimulate activation of phagocytes and ‘help’ B cell antibody production and effector CD8 T cell responses. APCs displaying MHC class II uptake microbes from the extracellular environment in endocytotic vesicles which fuse with lysosomes causing protein degradation. MHC class II molecules are synthesised in the endoplasmic reticulum and dispatched in vesicles which fuse with these phagolysosomes on their path to the cell surface [71]. At this stage MHC molecules are associated with a protein named invariant chain (Ii) which blocks the peptide binding groove and ensures stability of the molecular complex [72]. When the vesicles containing the MHC-Ii complex and peptide products fuse, enzymes degrade the invariant chain leaving a small protein CLIP (class II associated invariant chain peptide) in the antigen binding groove. A protein named
H2-M or H2-DM (in mice) or HLA-DM (in humans) removes CLIP and allows the MHC to bind antigenic peptides within the lysosome [73]. This stable MHC-antigen complex is then displayed on the cell surface and elicits appropriate phagocytic, cytotoxic and antibody responses via CD4 T cell activation. This forms the *exogenous pathway* [70].

It should be noted that the resultant immune response is exquisitely targeted towards elimination of either extracellular or intracellular pathogens via these mechanisms: intracellular pathogens require killing via cytotoxic cells (CD8 T cells), whereas extracellular pathogens are killed via enhancement of the innate immune response, activation of phagocytes and the production of antibodies which are orchestrated by CD4 (helper) T cells. While it is convenient to delineate the presentation of antigens in this way, in reality extracellular antigens can be presented on MHC class I molecules in a process known as ‘cross-presentation’ [74]. Vesicles which have taken up extracellular pathogen can redirect their cargo to proteasomes, breaking down the protein to peptides which are then transported to the endoplasmic reticulum for association with newly formed MHC class I molecules. Cross-presentation is important for priming cytotoxic responses to tumours and pathogens which aren’t directly expressed [75].
1.3 Role of T Cells

‘I find it astonishing that the immune system embodies a degree of complexity which suggests some... analogies with the human language, and that this cognitive system has evolved and functions without assistance of the brain’ - Neils Jerne

1.3.1 The T cell receptor complex

The T cell receptor (TCR) is a heterodimer consisting of polypeptide chains. Most T cell receptors are of the αβ subtype, being composed of an α chain and a β chain [76]. Each chain is composed of a N-terminal variable domain (V), a constant domain (C), a hydrophobic domain which spans the cell membrane and an internal cytosolic domain [77]. The V portions contain three hypervariable regions which are directly involved in recognising antigen in association with MHC and are referred to as complementarity determining regions (CDR) [78]. T cell receptors are formed through somatic recombination of germline genes. The β chain contains variable (V), diversity (D), joining (J) and constant (C) genes, while the α chain does not contain a D segment [79].

Recombinase-activating genes (RAG-1 and RAG-2) splice out nucleotides between V, D and J segments allowing rearrangement to produce unique RNA products [80]. Combinational diversity is achieved through random selection of different gene segments, and this can be further amplified by introduction of different nucleotides at the junctions where they combine (junctional diversity). This process allows for the formation of an enormous repertoire of receptors capable of reacting to the range of pathogens we encounter during our lifetime.
The *T cell receptor complex* is composed of the T cell receptor (which facilitates antigen recognition), two associated invariant CD3 protein heterodimers and one ζζ homodimer. The cytosolic portion of the TCR is too short to allow it to transmit signals to the cell. The CD3 and ζζ proteins are non-covalently bound to the TCR through charge interactions and provide the capability for signal transduction via phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) when the T cell receptor is activated (see Figure 3) [81]. There are also a number of *accessory molecules* which play a vital role in the activation of transcription factors: these include the co-receptors (CD4 and CD8), co-stimulators, inhibitors and adhesion molecules. Co-stimulation is provided via a range of molecules, but CD28 (which is expressed on naïve T cells and binds to CD80 (B7-1) and CD86 (B7-2) on APCs) is the most well-known [82]. Activated T cells also express also CD278, otherwise known as ICOS (inducible T cell costimulator), which binds to the family of B7 ligands [83]. The tumour necrosis factor receptor (TNFR) family also provide co-stimulation: molecules involved include CD40L (which binds to CD40 on APCs), OX-40, 4-1BB, CD30 and CD27 [83]. Adhesion is mediated through such molecules as leukocyte function-associated antigen 1 (LFA-1) and very late antigen 4 (VLA-4) which bind to the intercellular adhesion molecules 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), respectively, and act to stabilise T cell and APC binding [84]. Negative regulation of the immune response is provided by cytotoxic T lymphocyte antigen 4 (CTLA-4) which binds to B7 on APCs and the programmed cell death protein 1 (PD-1) which binds to its ligands PD-L1 and PD-L2 [85, 86].
1.3.2 **PRIMARY ACTIVATION OF T CELLS**

T lymphocytes are known as MHC restricted in that they can only recognise peptide fragments displayed by MHC molecules [87]. T cells interact with the MHC molecule via the T cell receptor. A T cell receptor has dual specificity for peptide antigen and the polymorphic residues of the MHC molecule that is displaying that particular antigen. The CDR3 hypervariable region of the TCR binds to peptide whilst CDR1,2 bind to framework determinants of the MHC molecule (see Figure 4 [88]).
In most cases, naïve T cells require recognition of peptide in association with self MHC and the CD4 or CD8 co-receptor, costimulators (which may be cytokines or cell-surface receptors, signal 2) and exposure to survival cytokines (IL-1, IL-12, IFNα/β) in order to become activated [89, 90]. If a T cell comes into contact with an MHC-peptide complex but does not receive the costimulatory signal, it will usually undergo anergy or apoptosis [91-94]. Of note, T cell receptors with very high affinity for MHC-peptide have lesser requirements for co-receptor binding and costimulation.

A T cell receiving the necessary costimulatory signals will produce stimulatory cytokines such as interleukin-2, resulting in the cascade of processes which lead to clonal expansion, differentiation into effector cells and the formation of memory T cells. Antigen presenting cells which are capable of providing this co-stimulatory signal are known as professional APCs. The most well-studied costimulator is CD28 which binds to the ligands CD80 or CD86 on antigen presenting cells [82, 95]. CD 40 ligand (CD40L or CD154) displayed on effector T cells acts on target cells (APCs, B cells, dendritic cells and macrophages) to produce effector functions and enhance the
immune response [96]. CD40 stimulation results in B7 upregulation on APCs, plays a critical role in antibody production by B cells, and promotes adhesion molecule expression and production of inflammatory cytokines, hence playing a pivotal role in the orchestration of the immune response to foreign tissue [97-99].

When a TCR complex identifies cognate antigen in association with MHC, there is a clustering of co-receptors (CD4 or CD8) which contain Src family kinases on their cytoplasmic tail and phosphorylate the ITAMs on CD3 and ζζ [100]. This initiates a cascade of events including the activation of Syk family tyrosine kinase, phosphorylation of adaptor proteins and binding of signalling molecules. The migration of surface and intracellular signalling molecules, co-receptors and co-stimulators to the site of contact between the T cell and APC lead to cytoskeletal changes forming a supramolecular activation cluster (SMAC) or immunological synapse, reinforced by adhesion molecules [101]. The SMAC is important in regulating lymphocyte activation, enhancing signalling, directing cytokine secretion and terminating effector function [102-104]. Subsequent activation of the G proteins Ras and Rac stimulate the activation of a range of mitogen-activated protein (MAP) kinases and extracellular-signal-related and c-Jun N-terminal kinases, whilst activation of the phospholipase C-calcium pathway activates calcineurin and diacylglycerol activates protein kinase C [105]. Such enzymes lead to activation of transcription factors, including NFAT, NF-κB and AP-1 which regulate gene expression and mediate clonal expansion, differentiation and effector function.
1.3.3 Site-directed mutagenesis of MHC class I abrogates direct antigen recognition by CD8 T cells

Cytotoxic T cell binding requires recognition of polymorphic peptide residues by the TCR in association with class I MHC and binding of the CD8 co-receptor with its ligand. The polymorphic peptide residues which the TCR recognises are part of the α1 and α2 chains of class I MHC, whereas the CD8 co-receptor binds to an invariant portion of the α3 chain (see 1.2.4 Class I). In murine models, it has been shown that MHC class I recognition is dependent on the 227 position of the α3 chain encoding either glutamic acid or aspartic acid: mutation to lysine abrogates the ability of CD8-dependent T cells to prime a class I dependent immune response by eliminating CD8 co-receptor binding [60, 106, 107]. Therefore, by performing site-directed mutagenesis at this point, it is possible to create defective class I MHC which displays peptide but cannot prime a CD8 T cell response.

1.3.4 CD4 ‘helper’ cells

T cells are broadly characterised into helper (CD4) T cells and cytotoxic (CD8) T cells. While it is useful to consider helper T cells as involved in stimulating humoral immunity and phagocytic function and cytotoxic T cells as the primary eradicators of intracellular pathogens, in reality their relationship is co-dependent. CD4 T cells differentiate into subsets of helper cells which play important roles in cytokine secretion and the coordination of the immune response.

Interleukin-2 is produced from naïve CD4 T cells after activation and serves a primary role in stimulating proliferation and differentiation. Differentiation into a particular T
cell subset is dependent upon the cytokine milieu present during activation, and this in turn is dependent on the type of antigen present. For example, bacteria stimulate the production of IL-12 and interferon-γ (IFN-γ) by macrophages and natural killer cells during the innate immune response and this in turn drives CD4 cells towards a Th1 phenotype [108, 109]. Likewise, early production of IL-4 drives a Th2 response [110, 111]. The currently recognised T cell subsets are Th1, Th2, Th17 and Th22 (as well as Tregs, see 1.3.6 Regulatory T cells): each of this is induced by and produces a different array of cytokines [112].

Th1 cells produce interferon-γ which activates macrophages and immunoglobulin production, whereas Th2 cells produce interleukin-4, 5 and 13 which result in mast cell and eosinophil activation and B cell class switching to produce IgE: additionally, the cytokine response of each subset inhibits the other [113, 114]. Th17 cell differentiation is initiated by TGF-β and production of IL-6, 21 and 23 by APCs [115]. These cells produce IL-17 and IL-22 which act to recruit neutrophils and monocytes, increasing the inflammatory response and the production of antimicrobial peptides [116]. In general terms, Th1 cell populations are effective against intracellular pathogens, the Th2 subset is optimised for eradication of parasites and the Th17 response targets extracellular microbes. More recently defined helper populations include Th22 cells which secrete IL-22 and play a role in microbial defence at mucosal surfaces, and Th9 cells which secrete IL-9 and are thought to protect against helminth infection [117, 118]. The cytokines inducing subset differentiation, the major transcription factors involved and the cytokines produced by mature CD4 effector cells are shown in Table 3 [119-122].
Table 3: CD4 T cells subsets

<table>
<thead>
<tr>
<th>Subset</th>
<th>Inducing cytokine</th>
<th>Transcription Factors</th>
<th>Cytokines Produced</th>
</tr>
</thead>
<tbody>
<tr>
<td>Th1</td>
<td>IL-12, INF-γ</td>
<td>STAT1,4, T-bet</td>
<td>INF-γ</td>
</tr>
<tr>
<td>Th2</td>
<td>IL-4</td>
<td>STAT6, GATA-3</td>
<td>IL-4,5,13</td>
</tr>
<tr>
<td>Th9</td>
<td>IL-4, TGF-β</td>
<td>PU.1, Spi-1, IRF4</td>
<td>IL-9,10</td>
</tr>
<tr>
<td>Th17</td>
<td>TGF-β, IL-6,21,23</td>
<td>STAT3, RORyt</td>
<td>IL-17,21,22</td>
</tr>
<tr>
<td>Th22</td>
<td>IL-6, (IL-23)</td>
<td>T-bet, AhR</td>
<td>IL-22</td>
</tr>
<tr>
<td>Treg</td>
<td>IL-2, TGF-β</td>
<td>STAT5, FoxP3</td>
<td>TGF-β, IL-10</td>
</tr>
</tbody>
</table>

1.3.5 CD8 ‘CYTOTOXIC’ CELLS

Cytotoxic CD8 cells are the primary effectors of cell-mediated immunity and play an important role in the rejection of allografts. Helper T cells play an important role in priming naive CD8 T cell as well as their expansion and differentiation into effector and memory pools. CD40-CD40L binding between helper T cells and antigen presenting cells ‘licence’ the APCs, which then provide help signals to cytotoxic T cells via CD27-CD70 interactions, co-stimulators and cytokines, enhancing proliferation and producing functional memory pools [123-126].

An effector CD8 T cell causes death of target cells through perforin-dependent and perforin-independent mechanisms, both of which induce apoptosis [127]. After antigen recognition, there is exocytosis of lytic granules containing perforin and granzymes A and B in association with serglycine. Perforin is thought to perform a role in establishing pores in the membranes of target cell facilitating the entry of granzyme B which activates the caspase cascade and granzyme A which directly cleaves nuclear proteins [128, 129]. Alternatively, lytic granules may enter the target cell via mannose-
6-phosphate receptor-mediated endocytosis [130]. Apoptosis via the perforin-independent pathway is mediated by direct cell contact: binding of Fas ligand on CD8 cells with Fas on target cells activates the caspase cascade inducing apoptosis [131]. Furthermore, secretion of cytokines such a TNFα can activate the caspase cascade, whereas secretion of IFN-γ leads to increased expression of MHC class I and Fas which augment cytotoxic killing [132].

1.3.6 REGULATORY T CELLS

Gershon and his colleagues initially postulated the existed of a population of T cells able to suppress immune responses in 1972, which they term suppressor T cells [133]. Studies of cardiac allograft survival in rat models exposed the existence of a suppressor T cell population which was CD4+ and dependent on IL-2 [134-137]. In 1997, Groux et al. described a clone of antigen specific T cells termed type 1 regulatory T cells (Tr1) which actively suppressed CD4 T cell proliferation in a murine model of colitis [138]. The most well-characterised of the Tregs are a subset of CD4 cells which express the α-chain of the IL-2 receptor (CD25), referred to as CD4+CD25+ regulatory T cells. Suggested in 1995, it wasn’t until 2000 that this population was identified [139-141]. An additional subset of regulatory T cells, known as Tr3, was reported after investigation of TGF-β secreting suppressor cells in animal models of experimental autoimmune encephalitis [142]. In fact, much of the ability of Tregs to suppress immune responses is mediated through the secretion of transforming growth factor-β [143, 144]. It has since become increasing clear that the regulatory T cell (Treg) population is a diverse and pivotal element of the immune system, maintaining self-tolerance and providing negative feedback to immune responses [145, 146].
display a vast array of chemokine receptors and adhesion molecules which distribute them to sites of inflammation, tumours and transplants [147]. They are produced either in the thymus or peripheral lymphoid organs. Thymic generation of regulatory T cells from immature CD4 T cells is now recognised as one of the central pathways that maintains self-tolerance (see 1.8.1 Central tolerance mechanisms). In the periphery, Tregs are thought to result from chronic antigen exposure without a corresponding innate immune response, starving the cells of appropriate cytokines and co-stimulation. Interestingly, it has been shown that Th17 cells and Tregs are closely related and that these phenotypes are not fixed, but display plasticity depending upon the composition of the extracellular milieu [148]. Accordingly, expression of IL-2, IL-6 and IL-1β can change Tregs to a Th17 phenotype, whereas IL-27 induces cytopathic Th17 cells to produce IL-10 which is immunosuppressive [149, 150].

Research into human autoimmune disease led to the critical discovery of the forkhead box P3 (FoxP3) transcription factor which has played a crucial role in our understanding of regulatory T cells [151]. It has been shown that FoxP3 is necessary for the differentiation, proliferation, suppressor function and maintenance of regulatory T cells [152-154]. Induction of FoxP3 occurs in both developing immature T cells and mature peripheral T cells, leading to the formation of naturally occurring/thymic Treg (nTregs) and inducible Treg cell populations (iTregs) [155]. IL-2 and TGF-β play an important role in inducing the transcription of FoxP3 and hence the formation of Tregs as well as effecting their suppressor function [156]. TCR antigen binding during thymic maturation has been shown to be important in directing CD4 T cells to differentiate into T regulatory cells [157]. While the affinity of antigen binding appears to play a role in the induction of FoxP3 expression, the duration of binding
dictates specific epigenetic changes [158-160]. Recent investigation has suggested that the development and maintenance of regulatory populations is complex and relies not only on FoxP3 but also on the induction of these stable Treg-cell-specific epigenetic patterns including demethylation within the FoxP3, CTLA-4 and Eos loci as well as extrinsic stimulation [160-164]. Of the regulatory T cell population, the CD4⁺CD25⁺FoxP3⁺ subset are the most extensively studied. CD4⁺CD25⁺FoxP3⁺ nTregs represent 5-10% of peripheral CD4 T cells [165]. Adaptive or induced Tregs (iTreg) arise from FoxP3 expression in peripheral CD4⁺CD25⁻ or CD4⁺CD25⁻ T cells [166, 167].

The suppression of immune responses by Tregs is accomplished by secretion of cytokines such as IL-10 and TGF-β as well as inhibition of T cell interactions with antigen presenting cells. Binding of interleukin-10 results in the activation of STAT3 (signal transducer and activator of transcription 3), causing inhibition of activated macrophages and dendritic cells by interfering with IL-12 production as well as the inhibition of co-stimulators and MHC class II molecule display [168]. TGF-β binding results in the activation of SMAD (mother against decapentaplegic) transcription factors which have a number of important immunological effects. These include inhibition of proliferation and function of T cells, inhibition of macrophages and promotion of tissue repair via stimulation of collagen synthesis. Not only do Tregs inhibit APC activation and function, tumour models have also demonstrated APC killing via cytolyis [169, 170]. In vitro studies have underscored the importance of cell contact, demonstrating that Tregs can induce cytolyis through secretion of perforin and granzymes or apoptosis via expression of galectin-1 [171-174]. Additionally, CD25⁺ Tregs avidly consume IL-2 while suppressing its production in effector cells, leading to T cell apoptosis through starvation [175].
CTLA-4, expressed by Fox3P3⁺ Tregs, has been shown to be necessary for their regulatory function and is thought to work by reducing expression of B7 costimulatory molecules and induction of immunosuppressive enzymes such as indoleamine 2,3-dioxygenase (IDO) which depletes extracellular tryptophan stores and produces pro-apoptotic metabolites [169, 170, 176]. CTLA-4 binds B7-1 and B7-2 with higher affinity than B7, restricting costimulatory signals. T cell activation is further reduced by Treg expression of lymphocyte-activation gene 3 (LAG-3), fibrinogen-like protein-2 (FGL2) and neuropilin-1 (Nrp-1) [165]. The inhibition of APCs and reduced expression of costimulatory ligands B7-1 and B7-2 is augmented by binding of LAG-3 to MHC class II and FGL2 to the inhibitor receptor FcγRIIB (CD32), while Nrp-1 amplifies Treg-APC interaction [177-179].

The central role of Tregs in controlling immune responses makes them attractive targets for immunotherapy. The ability of Tregs to mediate the normal immune response is targeted towards the particular arm of the immune system implicated. Experimental studies have shown that induction of T-bet, IRF-4 (interferon regulatory factor-4) or STAT3 transcription in Tregs corresponding to Th1, Th2 or Th17 responses results in unchecked and deregulated immune responses, likely through defective migration, function and homeostasis [180-182]. Further understanding of the pathways involved in Treg activation and recruitment will play an important role in transplantation immunology and the pursuit of operational tolerance.
1.3.6 MEMORY T CELLS

Sir Peter Medawar’s skin transplant experiments demonstrated that the immune system displays memory; that is, re-exposure to the same immunogen elicits a rapid, specific response [34, 35]. Memory is the hallmark of the acquired immune response. Memory cells are composed of CD4, CD8 and B cell subsets produced during antigen exposure and are long-lived cells which exist in a quiescent state. Their survival is dependent on IL-7 and IL-15 which strengthen anti-apoptotic pathways (Bcl-2, Bcl-XL) and hence promote long-term cell survival [183, 184]. Human memory T cells express CD45RO (CD44 in mice), CD2 and CD11a [185, 186]. Two populations, effector ($T_{EM}$ cells) and central ($T_{CM}$ cells), have been identified [187]. Central memory T cells display CCR7 and CD62L homing receptors which enable them to migrate between the spleen and lymphoid organs, whereas $T_{EM}$ cells downregulate these same receptors enabling them to provide surveillance in peripheral tissues [188-191].

Upon antigen re-exposure, $T_{EM}$ cells rapidly initiate an effector response, whereas $T_{CM}$ cells undergo rapid clonal expansion to generate additional effector cells. $T_{CM}$ cells are less dependent on co-stimulation, more responsive to antigen, and show a greater propensity to upregulate CD40 ligand; $T_{EM}$ cells display chemokine receptors which allow them to quickly navigate to areas of inflammation and provide effector responses either directly as CD8 cells or via secretion of cytokines as CD4 cells [188]. Memory B cells are termed either protective or reactive, depending on their ability to immediately secrete antigen-specific antibodies or rapidly proliferate and differentiate into plasma cells [192, 193]. Pools of briskly reactive memory cells sensitised to antigenic peptides on transplanted organs pose a difficult barrier to transplant tolerance.
1.4 ROLE OF B CELLS

‘We can remove antibodies from someone who’s in the middle of a terrible rejection, and save those kidneys’ - Robert Montgomery

B cells arise from lymphoid progenitors in the bone marrow and are vitally important for eradicating extracellular pathogens in the tissues and the bloodstream through the production of antibodies. Their importance is underscored by primary immunodeficiency syndromes resulting from defects in antibody production such as common variable immunodeficiency (acquired hypogammaglobulinaemia) which render the host susceptible to recurrent bacterial infections. They also play a prominent role in hyperacute and chronic allograft rejection (see 1.6 Rejection – the alloresponse).

Antibodies are composed of two light chains and two heavy chains joined by disulphide bonds into a Y-shaped molecule (see Figure 5, based on [194]) [195]. Each light chain is composed of variable (V_L) and constant (C_L) regions, while heavy chains are composed of one variable (V_H) and three or four constant regions (C_H1-4) [196]. All variable regions contains three hypervariable complementarity determining regions (CDRs) with CDR3 (at the junction of the V and C regions) containing the most diversity and therefore playing the major role in antigen binding [197]. Each ‘arm’ of the Y, composed of a light chain and the variable and first constant region of the heavy chain, is responsible for binding antigen and it is therefore determined the fragment of antigen binding (F_ab). The remaining portion of the immunoglobulin molecule is a
constant region which performs effector function and transmits biological activity, denoted the \textit{fragment crystalline} (F\textsubscript{c}) [196].

The immunoglobulin genes encode two light chains (\(\kappa\) and \(\lambda\)) and five heavy chains (\(\mu\), \(\delta\), \(\gamma\), \(\varepsilon\) and \(\alpha\)). Similar to T cell receptors, the heavy chain and the light chain are formed through somatic recombination of gene segments [198, 199]. The type of heavy chain delineates the class of the antibody produced and such antibodies may exist in different configurations (IgE is a dimer whereas IgM is a pentamer). Naïve B cells express IgM and IgD, however on antigen stimulation and signalling ‘help’ from CD4 T cells, they may change heavy chain isotype: this is known as \textit{class switching} [200]. The B cell receptor complex is comprised of membrane associated antibody and non-covalently linked proteins (I\(\alpha\) and I\(\beta\)) which transduce signals to the lymphocyte [201]. Activation requires cross-linking of membrane antibodies by multivalent antigen, resulting in phosphorylation of the I\(\alpha\) and I\(\beta\) cytoplasmic ITAMs by Src family kinases and subsequent enzymatic expression and production of transcription factors which regulate gene expression [202].
Figure 5: The B cell receptor complex

(Adapted from Abbus, Lichtman & Pillai, 2012)

1.5 ALLORECOGNITION

‘Don’t look for recognition without recognising yourself first’ - Ronald Dominguez

The immune response to transplantation between histoincompatible individuals leads to rejection, mediated through two primary steps: allorecognition and the alloresponse (also known as afferent and efferent arms, respectively) [203]. Allorecognition refers to the sensitisation of the immune system to alloantigens, while the alloresponse encapsulates the effector mechanisms which target the graft.
Allorecognition of foreign MHC class II by recipient helper T cells is critical in initiating rejection, which is largely effected via direct recognition of donor MHC class I by cytotoxic T cells with subsequent destruction of allograft tissue.

The primary alloantigens are donor major histocompatibility molecules and indeed it was the phenomenon of transplant rejection that led to the discovery of the MHC gene complex [203]. Minor histocompatibility antigens (miH) also play a role: these are processed cellular antigens encoded by sex chromosomes (H-Y antigen), autosomes or mitochondrial DNA [204]. The intensity of the immune response to transplantation is dependent on the degree of histocompatibility mismatch as well as which antigens are involved. In mice, differences in class I MHC molecules between donor and recipient promote rapid organ rejection, whereas class II molecule disparities elicit a more indolent response. In humans, it seems that whilst rejection is largely driven by class I antigen disparity, this is not a universal rule. For human kidney allografts, it appears that HLA-DR incompatibility is the most important initially (within the first 6 months), followed by HLA-B within the first few years and HLA-A long term [205, 206]. Furthermore, minor histocompatibility antigens usually elicit a low-grade immune reaction in cases of MHC-compatible donors; however if multiple miH mismatches are present the response can be vigorous [207, 208]. It seems that the relative importance of miH is also dependent on the organ transplanted: in cardiac allografts most responses are against MHC, in corneal allografts the response is against miH and in skin transplants the response is mixed (with a predominant MHC response) [209]. The immunological response is dominated by activation of naïve and memory T cells [165]. Antibodies can be formed against donor MHC class I and class II antigens, however they can also be directed against MHC class I-related chain A (MICA) antigens, platelet
antigens, molecules of the renin-angiotensin system and polymorphic chemokines [210].

Three pathways of allore cognition are widely recognised: direct, semi-direct and indirect (see Figure 6) [211]. In the direct pathway, intact donor MHC molecules are directly recognised by host lymphocytes [212]. It is thought that the donor MHC molecule (with or without donor peptide), is recognised by the host as self-MHC associated with foreign peptide [213-215]. After transplantation, donor dendritic cells migrate to secondary lymphoid organs via the lymphatics and subsequently initiate an immune response via the direct pathway of allore cognition. Additionally, infiltrating host lymphocytes can recognise donor APCs within the grafted organ. The direct pathway of antigen recognition is therefore able to be activated rapidly, partially explaining its dominant role in acute rejection (see 1.6.3 Acute rejection). The important role of donor APCs in priming the immune response was confirmed in studies of renal allografts placed into histocompatible hosts prior to transplant to allow depletion of donor APCs. This resulted in long-term survival when these grafts were subsequently transplanted into mismatched animals: rejection could then be instigated upon introduction of donor dendrocytes [216]. In time, donor APCs die and host APCs migrate to the grafted tissue and process donor alloantigens in the graft, perpetuating the immune response via the indirect pathway [217]. Conversely, donor DCs are short-lived in the recipient and do not participate significantly in chronic rejection.
The indirect pathway follows the normal processing of microbial peptides. A number of the studies in the 1990’s showed that host dendritic cells migrate to the graft and pick up alloantigens (donor MHC molecules) before returning to the secondary lymphoid organs to interact with host lymphocytes [218-220]. The catabolism of donor MHC molecules by host APCs and their subsequent display as foreign peptides in association with class II MHC stimulates an immune response [213, 214, 221-223]. This mechanism is dominant in chronic rejection and is also the process by which minor histocompatibility alloantigens are recognised [224]. The resulting activation of CD4 T cells is vital in helping CD8 T cells and B cell antibody production [225]. Due to cross-presentation, donor peptides can also be displayed on class I self-MHC leading to the activation of CD8 cells via the indirect pathway [226, 227]. In a murine skin transplant model the population of CD8 T cells activated through cross-presentation was similar in size to the indirectly activated CD4 population and contributed to rejection via the
destruction of vascular endothelium [228, 229]. There is an inherent temporal delay in activating the indirect pathway, and it remains a primary mediator of chronic rejection (see \textbf{1.6.4 Chronic rejection}). Unlike the direct pathway, the indirect pathway is mediated by a limited number of T cell clones restricted to dominant determinants of donor MHC [221, 230-232]. With time the number of dominant antigen determinants that are recognised increase [233-235]. Such ‘antigen spreading’ is thought to occur from the presentation of previously concealed peptide determinants due to changes in processing and display as a result of the inflammatory cytokine milieu [212]. This is thought to contribute to chronic rejection, where a continued immune-mediated destruction of the allograft is maintained.

The semidirect pathway involves host dendritic cells acquiring intact donor MHC molecules from donor dendritic cells and subsequently initiating an alloimmune response [236, 237]. This phenomenon of donor MHC transfer is known as \textit{cross dressing} and may occur via transportation of MHC molecules in exosomes or by membrane exchange during direct cell contact [238, 239]. In this way, the same APC can recruit both CD4 and CD8 T cells, providing an explanation for how help signals can be provided for complete CD8 activation.

The precursor frequency of alloreactive cells is very high. It is estimated that 5-10% of a host’s T cells respond to alloantigens via direct allorecognition, explaining why the immune response to transplantation is extremely pronounced [212, 240]. Multiple theories exist as to how such a large subset of the T cell repertoire is able to respond: the two best characterised are the \textit{multiple binary complex} and \textit{high determinant density} models [241, 242]. The multiple binary complex model proposes that a large
array of peptides bound to a single allogeneic MHC product can activate multiple alloreactive T cells [203, 243]. The high determinant density theory proposes that if alloreactive T cells are capable of recognising donor MHC molecules, then the density of antigen display is high and even T cells with low receptor affinity can be activated [215]. Both likely contribute to the ability of the host’s immune system to avidly recognise donor tissues.

Activation of native memory T cells can perpetuate a vigorous targeted reaction and remain a significant barrier to transplant tolerance in the human population. Alloreactive memory T cells may exist from prior exposure to alloantigen (previous transplant), blood transfusion or pregnancy [165]. They may also arise from molecular mimicry following microbe exposure (heterologous immunity) or homeostatic proliferation [244]. Importantly, memory T cells can become activated without co-stimulation and are also more resistant to death via upregulation of anti-apoptotic molecules [245, 246]. They elicit a powerful and rapid response to transplanted tissues and their phenotype imparts a greater resistance to clinical immunosuppressive regimes such as pharmacotherapy, T cell depletion through monoclonal antibody stimulation or co-stimulatory blockade [120, 247-249].
There are four widely recognised profiles of rejection termed hyperacute, accelerated, acute and chronic rejection. These are based on the temporal relationship between time of transplant and organ damage, with each occurring due to primarily different immunological processes (see Table 4).

T cells have a pivotal role in transplant rejection through recognition of donor antigens and orchestration of an effector response including secretion of cytokines and cytotoxic killing of donor cells [250]. This is exemplified by that fact that animals deprived of T cells will not reject allografts (unless they have pre-existing antibodies to donor antigens) [251]. Activation of macrophages and autocrine stimulation of CD4 T cells leads to a delayed hypersensitivity reaction with expression of tumour necrosis factor and reactive oxygen species, as well as stimulation of cytotoxic CD8 T cells and B cells. Of note, while B cells require CD4 help to be fully activated to protein antigens, B cells also play a role in T cell survival and proliferation in their role as APCs [225, 252]. CD4 T cells are critical to allograft rejection and elicit a T-helper response (T_{H1}, T_{H2} or T_{H17}) dependent on the local cytokine milieu. The T_{H1} response leads to production of IFN-γ and IL-2, resulting in stimulation of cytotoxic T cells, delayed-type hypersensitivity, synthesis of IgG2a by B cells and subsequent activation of complement [165]. They also express Fas-ligand which can induce cell cytolysis. The T_{H2} response causes the release of multiple interleukins (IL-4,5,9,10 and 13) which
active eosinophils and B-cells: \( T_{\text{H}2} \) responses with graft eosinophil infiltration can be seen in murine cardiac and human liver allografts [253, 254]. \( T_{\text{H}17} \) cells producing IL-17 promote neutrophil recruitment and have been noted in animal models, especially in the context of acute rejection [255]. CD4 T cells orchestrate the effector response involving the innate immune system, antibodies, complement and cell-dependent cytotoxicity. Graft rejection can be mediated by any of these three pathways of CD4 activation. Activated alloreactive CD8 T cells play a prominent role in acute rejection via the direct pathway (see 1.5 Allorecognition). In renal allografts, cytotoxic CD8 cells containing perforin and granzymes A and B or expressing Fas ligand are commonly found [256]. Such T cells directly destroy donor tissue. The final outcome to transplant depends on the balance of cytotoxic to immunosuppressive T cells and the surrounding cytokine environment; however, as the process of transplantation is pro-inflammatory, cytokines secreted during transplant will favour a destructive phenotype.

B cells augment T cell survival and proliferation in their role as APCs, while requiring CD4 help to become fully activated to protein antigens and produce antibodies [225, 252]. The coincidence of antibody-mediated rejection (AMR) with cell-mediated responses is becoming increasingly apparent. Although the role of antibodies in hyperacute rejection has been recognised for many years, more recently evidence for humoral mechanisms in acute and chronic rejection are emerging. Binding of donor-specific antibodies (DSA) to alloantigens (particularly donor vascular endothelium) activates the complement and kinin pathways leading to inflammation, cell injury and activation of platelets and the clotting cascade [257]. Antibody-dependent cell-mediated cytotoxicity and antibody-endothelial reactions leading to increased Von
Willibrand Factor expression and upregulation of cell adhesion molecules are also seen [258-260].

1.6.1 HYPERACUTE REJECTION

Hyperacute rejection of an allogeneic transplant usually occurs within minutes of re-establishing the blood supply to the graft. It occurs due to the presence of pre-existing donor-specific antibodies in the recipient secondary to pre-sensitisation as a result of prior transfusion, previous transplantation or pregnancy [261]. DSAs are usually IgG targeted against HLA antigens or IgM targeted against blood group antigens [203]. These circulating antibodies bind to alloantigens on the endothelium of donor vessels, activating clotting cascades and the complement system causing inflammation (vasculitis), edema and thromobosis with resultant ischaemia and necrosis [257]. Histological examination of organs after hyperacute rejection show arteritis, interstitial oedema and necrosis [261]. Clinically, the organ appears mottled, dusky and oedematous. The universal use of pre-transplantation cross-matching has made hyperacute rejection a rare occurrence in modern times.

1.6.2 ACCELERATED REJECTION

Accelerated rejection is composed of two subtypes, both of which require previous sensitisation of the recipient to graft antigens. The first of these is antibody-mediated, and occurs as a more indolent form of hyperacute rejection in the setting of low titre DSAs. The second is the reactivation of sensitised effector T cell populations resulting
in rapid graft destruction, the process originally described as second-set rejection by Sir Peter Medawar.

1.6.3 Acute rejection

Acute rejection remains the primary cause of early graft failure, occurring days to weeks after transplant. Histological examination of the graft reveals both activated and memory subtypes of cytotoxic T lymphocytes, the primary effectors of acute rejection [262]. This rapid and vigorous reaction is dependent on the direct pathway of alloantigen presentation and the activation of memory cells sensitised to antigens which mimic donor MHC (see 1.5 Allorecognition). The activation of T lymphocytes results in direct cell killing and the activation of inflammatory pathways resulting in endothelialitis in the vascularised graft [263].

Donor specific antibodies, either pre-formed or produced post-transplant, also play a role in acute rejection [264]. Acute antibody mediated rejection occurs in 5-7% of transplants and is seen in 20 - 48% of cases of acute rejection in pre-sensitised positive cross-match patients, the rest resulting from T cell mediated processes [265, 266]. Histological examination reveals deposition of complement components and antibody-mediated vascular injury, with neutrophilic infiltration, oedema, and thrombus formation [266, 267].
1.6.4 **CHRONIC REJECTION**

Chronic graft rejection is now the leading cause of graft failure, characterised by an indolent loss of graft function occurring over months to years. The loss of organ function is thought to occur from alloantigen dependent and independent mechanisms, including damage secondary to IRI in the peri-transplant period, a chronic low-grade host immune response, cumulative damage from previous episodes of acute rejection and in the case of renal transplant, nephrotoxicity due to immunosuppressives. T cells reacting against graft antigens release cytokines which induce the proliferation of vascular smooth muscle cells and fibroblasts, resulting in fibrosis and graft arteriosclerosis [268]. In kidney transplant, the classic findings of chronic allograft nephropathy are parenchymal fibrosis, atrophy of tubules, vascular occlusion and glomerulopathy, while the findings in lung transplantation are thickened small airways characteristic of bronchiolitis obliterans syndrome. Graft arteriosclerosis is a progressive phenomenon; in renal allografts it is seen in 5% at 1 year and 20% at 5 years [269].

Antibodies also participate in chronic rejection. In kidneys, they cause chronic allograft glomerulopathy characterised by duplication of the basement membrane in glomerular and peritubular capillaries with mesangial expansion [270, 271]. Clinically, patients may be asymptomatic or they display marked proteinuria, hypertension and graft dysfunction [257].
<table>
<thead>
<tr>
<th>Rejection</th>
<th>Time</th>
<th>Effectors</th>
<th>Mechanism</th>
<th>Histology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperacute</td>
<td>Minutes → hours</td>
<td>Preformed alloantibodies</td>
<td>Binding to donor endothelium</td>
<td>Thrombotic occlusion of vessels</td>
</tr>
<tr>
<td>Accelerated</td>
<td>Days → weeks</td>
<td>Preformed alloantibodies (low titre) AND/OR Sensitised T cells</td>
<td>As for hyperacute rejection but less vigorous AND/OR CD8 killing Cytokine release ADCC</td>
<td>Thrombotic occlusion Parenchymal damage and inflammation Vessel necrosis</td>
</tr>
<tr>
<td>Acute</td>
<td>Days → months</td>
<td>T cells B cells</td>
<td>CD8 killing Cytokine release Antibody binding to endothelium ADCC</td>
<td>Parenchymal damage Interstitial inflammation Transmural vessel necrosis</td>
</tr>
<tr>
<td>Chronic</td>
<td>Months → years</td>
<td>T cells Drugs</td>
<td>Cytokine secretion Vascular smooth muscle proliferation Fibrotic repair after episodes of acute rejection</td>
<td>Arteriosclerosis Ischaemic damage Fibrosis</td>
</tr>
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Table 4: Profiles of rejection
1.6.5 **INNATE ALLOIMMUNITY AND ISCHEMIA REPERFUSION INJURY**

The innate immune response to transplanted tissue is termed *innate alloimmunity*. In 1994, it was postulated that reperfusion injury of renal allografts initiated acute rejection and contributed to the subsequent development of a chronic immune response [272]. This led to the formulation of the ‘injury hypothesis’, which postulates that oxidative injury to the allograft leads to the formation of damage-associated molecular patterns (DAMPS), also known as danger signals or alarmins, released by dying cells. Common DAMPS include HMGB1 (high mobility protein group B1), AGE (advanced glycation endproducts), amyloid-β peptides, heat shock proteins, hyaluronan fragments and the S100 family [273] (see Figure 7). DAMPS bind to a number of ligands such as toll-like receptors 2 and 4 displayed on intra-graft donor and recipient dendritic cells and the pattern recognition receptor RAGE (receptor for advanced glycation endproducts) encoded by MHC class III [274, 275]. Binding initiates the activation of transcription factors such as nuclear factor-kappa B (NF-κB), activator protein 1 (AP-1) and interferon regulatory factor 3 (IRF3) which drive the production of pro-inflammatory cytokines and the proliferation and maturation of dendritic cells, hence driving the innate immune response and potentiating adaptive immunity [276].

Costimulatory molecules have been shown to be of critical importance to the phenomenon of rejection, in particular the CD28/B7 system [277]. Such costimulators are upregulated in the context of ischemia reperfusion injury and the initiation of the innate immune response. There have been numerous studies examining the blockade of costimulatory pathways or the potentiation of inhibitory signals to induce operational tolerance (see **1.8 Achieving transplantation tolerance**).
Ischaemia reperfusion injury (IRI), donor brain death, cold preservation fluids and surgical trauma during transplantation lead to the generation of cytokines such as IL-6, TNF-α and IL-1β, mediating the migration of donor APCs to the draining lymph nodes where host lymphocytes reside [278-280]. Mature donor dendritic cells present alloantigen to recipient T cells driving the adaptive immune response [281]. This can elicit a vigorous immune reaction via direct allore cognition culminating in acute rejection. Natural killer cells can be stimulated to destroy allograft in one of two ways. NK cell inhibitory receptors recognise self class I MHC, providing a dominant negative signal which prevents activation. The lack of self class I MHC on foreign cells release NK cells from inhibition, thereby targeting donor cells for destruction (this is referred to as the ‘missing self’ theory) [282]. In this case, it is not strictly an alloantigen but
rather a lack of self-antigen which perpetuates the alloresponse. Additionally, stress ligands can activate NK cells through receptors such as NKG2D. IRI has been shown to lead to the upregulation of NKG2D ligands in the allograft after TLR-4 binding to HMGB1, which may potentiate destruction by NK and CD8 T cells [283].

1.6.6 REJECTION OF ALLOGENEIC SKIN GRAFTS

It has long been recognised that the integument in particular provides a strong antigenic stimulus. Current immunosuppressive treatments have little or no effect in skin transplantation, leaving isografting as the primary option for re-establishing an epithelial surface [212]. A large proportion of patients suffering burns or requiring reconstructive surgery would greatly benefit from the ability to achieve operational tolerance to allogeneic skin grafts. Dr. Joseph Murray, a plastic surgeon and transplant pioneer known for performing the first renal transplant in identical twins, recognised the potential benefits of skin allografts but believed that MHC-mismatched skin transplants would present an insurmountable barrier to graft acceptance [284].

Placement of an allogeneic skin graft elicits a vigorous immune response culminating in acute rejection. Skin grafts are densely populated with dendritic and Langerhans cells, conferring strong antigenicity [285, 286]. The innate immune system is activated initially leading to a plethora of responses: migration of neutrophils, macrophages and dendritic cells commences along with the secretion of pro-inflammatory cytokines and acute-phase reactants. After 3-4 days the lymphatics and blood supply are established: donor Langerhans cells and dermal dendritic cells migrate to the secondary lymphoid organs and activate alloreactive T cells via the direct pathway [212]. Numerous studies
with allogeneic or xenogeneic skin grafts have confirmed the presence of donor dermal antigen presenting cells (‘passenger leukocytes’) in the draining lymph nodes [287-291]. This migration of APCs through afferent lymphatics to the draining regional lymph nodes is vital to instigating the alloimmune response: studies of allografts suspended from the recipient bed by a vascular pedicle (thereby eliminating lymphatic drainage) indicate that such grafts do not reject [292-294]. The subsequent activation of T lymphocytes and the pathways through which they mediate rejection have been previously discussed (see 1.5 Allorecognition). Natural killer cells also participate in skin allograft rejection, however their role is complex and poorly understood. NK cells can elicit destruction of cells lacking donor class I MHC, or they can eliminate donor APCs, downregulate CD8 memory responses and promote tolerance [295-298].

1.7 SELF-TOLERANCE

‘In the practice of tolerance, one’s enemy is the best teacher’ - 14th Dalai Lama

Tolerance describes a process whereby the immune system recognises, but fails to mount a productive response against an antigen [299]. Tolerance to self-peptides is referred to as self-tolerance, the breakdown of which can cause the destruction of normal native tissues leading to the spectrum of autoimmune diseases. In healthy individuals, the immune system displays marked selectivity in targeting pathogenic microbes while remaining indifferent to self-peptides. Upon exposure to an antigen,
lymphocytes may become inactivated or destroyed instead of initiating an immune response. Antigens which induce tolerance are termed tolerogens. Tolerance can occur either centrally or peripherally. The primary mechanisms of tolerance include deletion, anergy, immunoregulation and clonal exhaustion [146]. Failure to recognise an antigen results in ignorance. Deletion refers to the destruction of self-reactive lymphocytes either centrally or peripherally, anergy corresponds to the functional inactivation of self-reactive T and B cells and immunoregulation is the ability of one population of leukocytes to control another [146, 300]. Clonal exhaustion occurs when chronic exposure to a particular antigen leads to either deletion or functional inactivation of the reactive lymphocyte population. Ignorance is seen when alloreactive lymphocytes do not encounter their cognate antigen, most commonly due to its existence at an immunoprivileged site.

Central tolerance occurs during maturation, either in the thymus (for T lymphocytes) or the bone marrow (for B lymphocytes). The process of tolerance is critical to confer immunological stability. Self-reactive lymphocyte may undergo negative selection via apoptosis (also known as clonal deletion), receptor editing, or be rendered anergic; furthermore, T cells may also undergo differentiation into a Treg phenotype which can to suppress the activation of self-reactive lymphocytes in the periphery [301, 302].

T cells undergo both positive and negative selection during development resulting in a repertoire of cells which recognise self-MHC and not self-peptides, hence conferring the property of MHC restriction. Low affinity recognition by immature T cells of self-MHC expressed on thymic cortical epithelial cells prevents programmed apoptosis, positively selecting them for self-MHC recognition [302]. Those which recognise self-
MHC with high affinity undergo clonal deletion or receptor editing while those that do not recognise self-MHC die by neglect [303, 304]. Expression of tissue-restricted self-antigens (TRAs) is mediated by medullary thymic epithelial cells, which provide an eclectic sample of self-peptides to developing T lymphocytes [305]. The transcription factor AIRE (autoimmune regulator) plays a critical role in expression of TRAs [306]. Immature T cells which bind self-peptides with high affinity display activation of the protein Bim initiating negative selection by apoptosis, or they may undergo differentiation into regulatory T cells (it is thought the avidity of binding may dictate the outcome) [303].

Because B cells are not MHC-restricted, they do not undergo positive selection during development. B cells expressing IgM which recognises multivalent self cell-surface molecules (such as MHC) undergo either receptor editing (re-arrangement of light chain genes via RAG) or apoptosis (clonal deletion) during development in the bone marrow [307, 308]. B cells which recognise soluble antigens downregulate IgM synthesis and are released into the peripheral circulation expressing IgD only, thus rendering them anergic, while those with receptor specific for non-crosslinking low avidity antigens remain ignorant [309]. B cells expressing both IgM and IgD which have not reacted to self-antigen in the bone marrow are released to encounter foreign antigen in the periphery.

For both B and T cells, a wide range of mechanisms and checkpoints exist to ensure that the selectivity of the immune system for pathogens is maintained. The mechanisms which maintain self-tolerance outside of the central lymphoid organs are collectively referred to as peripheral tolerance mechanisms. Not all self-reactive T
lymphocytes are eliminated in the thymus. Means of maintaining peripheral tolerance include anergy, suppression and deletion [310]. Exposure to self-antigen in the absence of co-stimulation, destruction of ITAMs by activation of cellular ubiquitin ligases such as Cbl-b, and engagement of inhibitory receptors such as CTLA-4 and PD-1 without adequate co-stimulation are pathways which are thought to result in loss of signal transduction and anergy [311, 312]. T cells which recognise self-antigens with high affinity without co-receptor stimulation activate the mitochondrial pathway of apoptosis via Bim, similar to negative selection during central tolerance induction [313]. Chronic antigen exposure results in expression of death receptors and their ligands, which triggers apoptotic death, often via the Fas/Fas ligand pathway (clonal exhaustion) [313].

B cells released into the circulation are either anergic (having already responded to self-antigen in the bone marrow), self-reactive (either ignorant or having evaded the mechanisms of central tolerance) or competent (not self-reactive and bearing antibodies for foreign peptides). B cell tolerance is maintained in the periphery largely through anergy and apoptosis. B cells which bind self-antigen strongly in the periphery undergo Bim-dependent deletion in the spleen [314]. Self-reactive B cells which bind self-antigen with low-affinity and have escaped central tolerance show decreased levels of the BAFF (B-cell activating factor) receptor (BAFF-R), conferring a survival advantage to healthy B cells (B cell development is critically dependent on the survival cytokine BAFF) [315]. Such cells are rendered anergic and maintain their quiescent state through constant exposure to self-antigen; they are usually located in the red pulp or extra-follicular areas of the spleen and are short-lived [316]. B cells which bind self-antigens very weakly or bear receptors for immunoprivileged self-antigens may
progress through maturation, however if subsequently exposed to their cognate antigen without appropriate T cell help they are deleted [317].

1.8 ACHIEVING TRANSPLANTATION TOLERANCE

‘Nothing dies so hard, or rallies so often as intolerance’ - Henry Ward Beecher

The holy grail of transplant immunology remains understanding and manipulating the mechanisms responsible for transplant rejection to achieve transplant tolerance. As discussed above (see 1.5 Allorecognition), the antigens of primary importance to graft rejection are the major and minor histocompatibility antigens. Tolerance to donor alloantigens involves multiple pathways and maintaining a state of tolerance is dependent on the persistence of alloantigen [318, 319]. Harnessing the inherent ability of the immune system to abort activation following exposure to antigens holds promise for inducing a state of operational tolerance, defined as ‘an antigen-specific unresponsiveness that is sustained in the absence of chronic immunosuppression’ [146]. It has long been recognised that exposure to a foreign antigen during foetal or early neonatal life can lead to tolerance to that antigen in adulthood, a phenomenon termed ‘neonatal tolerance’ [320]. This ability of the immune system to ‘tolerise to antigen’ diminishes with increasing age [321].

An understanding of the molecular mechanisms and pathways responsible for inducing tolerance has enabled researchers to explore avenues for achieving operational tolerance. Success will require a delicate balance of physiological
mechanisms and immunomanipulation [322]. The state of tolerance will be one in which there is a preponderance of regulatory T cells and a reduced population of alloreactive effector lymphocytes. It is currently felt that achieving tolerance will require an initial phase of deletion of alloreactive T cells, followed by a state of Treg dominance during which the remaining alloreactive T cells are suppressed (see Figure 8 based on [322]) [146]. The strategies employed generally focus on either central deletion or peripheral immunoregulation to achieve this goal. The unique ability of the liver to promote peripheral tolerance, known as ‘the liver tolerance effect’, may involve multiple interrelated mechanisms.

Figure 8: Achieving the balance of tolerance

(Adapted from Li, X.C. et al., 2001)
1.8.1 Central tolerance mechanisms

Approaches to inducing central tolerance rely on deletion as the primary mechanism [323]. Central self-tolerance mechanisms eliminate 90-99% of potentially autoreactive T cells during development, and some advocate that the large proportion of alloreactive cells in transplant recipients underscores the importance of central tolerance as a necessity for success [324]. Approaches to effecting central tolerance include the introduction of allogeneic cells into the thymus or the induction of mixed haematopoietic chimerism through myeloablative therapy and allogeneic bone marrow transplantation (BMT) with subsequent thymic seeding: such therapies aim to induce a state of central tolerance to donor antigens by disguising them as self-antigens. Mixed haematopoietic chimerism is seen after haematopoietic stem cell transplant where both allograft and recipient stem cells coexist. After allogeneic BMT, migration of host and donor marrow-derived cells into the thymus ensures ongoing central deletion of self and donor-reactive lymphocytes and the generation of T regulatory populations [325, 326]. Several animal studies including allogenic lung, skin, islet and renal transplant following myeloablative or non-myeloablative conditioning and induction of mixed chimerism have shown promise [327-331]. More recently, human renal allograft studies in HLA-matched and HLA-mismatched donors using non-myeloablative therapy for induction of mixed haematopoietic chimerism has shown that tolerance to transplants with subsequent removal of immunosuppression is potentially achievable [332, 333].
1.8.2 Peripheral Tolerance Mechanisms

Strategies to induce tolerance in the periphery rely heavily on anergy, immunoregulation and deletion [324, 334]. The various tolerance induction protocols include administration of monoclonal antibodies against T and B cells, costimulatory blockade, donor specific transfusion (DST), manipulation of regulator T cell function and harnessing the ‘liver tolerance effect’. Administration of immunosuppressive pharmaceuticals targets some of these pathways (see Table 5). Due to their pivotal role in the normal T cell response, interruption of costimulatory pathways or activation of negative regulatory pathways are attractive targets for immunotherapy. Costimulatory blockade prevents activation of T cells and promotes tolerance through the mechanisms of deletion, anergy and immunoregulation. Use of biological reagents such as abatacept (CTLA4-Ig) to block CD28:B7 interaction induces T cell unresponsiveness to alloantigens [94, 335]. Administration of CLTA4-Ig leads to blockade of B7 ligands and thereby prevents B7:CD28 binding, resulting in prolonged graft survival [336, 337]. The CD40:CD40L interaction, which is important for T cell and B cell activation as well as the upregulation of B7, has also received interest [98, 338, 339]. Depletion of T cells using anti-CD3 monoclonal antibodies and targeting CD20 on B cells with monoclonal antibodies to destroy them via apoptosis, ADCC and complement-dependent cytotoxicity are another methods used [340, 341].
<table>
<thead>
<tr>
<th>Major Pathway</th>
<th>Drug</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inhibit T cell proliferation</strong></td>
<td>Basiliximab</td>
<td>Anti-IL2α receptor antibodies: prevent IL2 stimulated proliferation</td>
</tr>
<tr>
<td><strong>Inhibit T cell activation</strong></td>
<td>Cyclosporin</td>
<td>Binds to cyclophilin: prevents calcineurin dependent dephosphorylation of NFAT and hence transcription of IL-2</td>
</tr>
<tr>
<td><strong>Costimulatory blockade</strong></td>
<td>Abatacept</td>
<td>CTLA4-Ig with a higher affinity for B71 then CD86: inhibits the co-stimulation necessary for activation and induces anergy</td>
</tr>
<tr>
<td><strong>T cell depletion</strong></td>
<td>Muronamab-CD3</td>
<td>Anti-CD3ε antibody: causes apoptosis of T cells</td>
</tr>
<tr>
<td><strong>B cell depletion</strong></td>
<td>Rituximab</td>
<td>Anti-CD20 antibody causes apoptosis of B cells, antibody-mediated and complement dependent cytotoxicity</td>
</tr>
</tbody>
</table>

Table 5: Examples of immunosuppressive pharmaceuticals

Donor specific transfusion of splenocytes given in conjunction with costimulatory blockade or immunosuppression has also been shown to extend allograft survival [342-344]. It is thought that alloantigens from donor cells are presented via the indirect pathway after uptake by recipient DCs, producing a population of anergic T cells which display hypo-responsiveness to these antigens [345].

It has become increasingly apparent that regulatory T cells, due to their ability to suppress and modulate the immune response, play a pivotal role in tolerance to alloantigens. As discussed previously, they can reduce secretion of cytokines, downregulate costimulatory molecules, eliminate effector cells or convert them into regulatory cells (infectious tolerance) [146, 346]. Regulatory T cells have been
demonstrated in grafts and their draining lymph nodes, positioned to impede both sensitisation and the effector response [347, 348]. Their numbers are increased in liver, renal and cardiac transplant patients who have achieved either stable graft function or operational tolerance [349-353]. Hall et al. demonstrated that transfer of CD4+CD25+ Tregs from rodents with prolonged allograft survival conferred graft tolerance upon other recipients [136]. Interestingly, induction of a regulatory population sensitised to a single donor alloantigen can inhibit the immune response to multiple antigens displayed by the same APC, a phenomenon known as linked unresponsiveness [354]. Such CD25+CD4+ Tregs have been shown to prevent rejection by both CD4 and CD8 T cell populations [355-358]. Once activated via TCR engagement with antigen, Tregs function to inhibit CD4 and CD8 activation and proliferation, modulate the effects of APCs and impede components of the innate immune system such as natural killer (NK) cells and mast cells [165, 359]. Recently-discovered ubiquitous, highly-conserved regions of IgG (in Fc and near Fab), dubbed Tregitopes, bind to CD4+CD25hiFoxP3+ nTreg TCRs and help regulate immune responses [360]. Tregitopes co-administered with antigen lead to the conversion to and/or expansion of antigen-specific iTreg populations [361].

1.8.3 THE PD-L1:PD-1 INTERACTION

Suppression of the immune response via the programmed cell death 1 ligand 1 (PD-L1), also known as CD274 and B7 homolog 1 (B7-H1), occurs in a variety of settings including transplantation, hepatitis, autoimmune disease and pregnancy and is an important component of peripheral tolerance (see Figure 9). Its ligand PD-1
(programmed cell death protein 1) is found on activated T cells, B cells, NK cell and macrophages where it is able to transmit inhibitory signals upon binding to PD-L1 [362]. PD-L1 is expressed more widely and is significantly upregulated in the context of IFN-Type 1 and IFN-γ secretion: it can be found on lymphocytes, macrophages, dendritic cells, epithelial cells and endothelial cells as well as the parenchyma of solid organs such as lung, liver and kidney [363, 364].

PD-L1:PD-1 interactions suppress TCR-mediated T cell proliferation; specifically, it inhibits phosphorylation of the ‘ZAP70/CD3ζ signalosome’, attenuating PKCθ activation and subsequently reducing IL-2 production [365]. There appears to be a balance between the strength of TCR and CD28 signalling and the inhibition imposed by PD-L1 ligation in determining the outcome of lymphocyte activation: PD-1 deficient mice readily develop a range of autoimmune diseases presumably due to a lowered threshold for T cell activation [364, 366]. PD-L1 is also important for maintaining tolerance to self-antigens, supressing the response of naïve self-reactive effector cells encountering their cognate antigen in association with dendritic cells [367, 368]. Studies with bone marrow chimeras into the role of PD-L1 in preventing diabetes in a mouse model revealed that PD-L1 expression on parenchymal cells is critical to preventing autoimmune disease, inhibiting self-reactive CD4 T cells and the production of INF-γ and TNF-α [369]. An additional role for PD-L1 has been noted in the induction of Tregs, augmenting FoxP3 expression: iTregs activated via the PD-L1 pathway showed increased efficiency of effector T cell suppression, especially at low iTreg/Teffector concentrations [370].
The importance of PD-L1 in maintaining tolerance by suppressing effector responses in the periphery has been repeatedly demonstrated. Tolerance to fully MHC-mismatched cardiac allografts in transgenic mice induced by CTLA4-Ig is broken by blockade of PD-L1 with increased circulating effector cytotoxic T cell and reduced intra-graft Tregs noted [371]. In a cold IRI liver transplant model in CL57BL/6 mice, PD-L1 expression was upregulated on sinusoidal epithelial and dendritic cells and induced on hepatocytes after transplant [372]. Repeating the transplants with PD-L1 KO donor livers resulted in increased graft necrosis and raised ALT levels, with amplified intra-graft CD8 infiltration and reduced pre-apoptotic CD8 cells. Mouse spontaneous liver allograft tolerance depends upon PD-L1:PD-1 interactions; tolerance is broken when liver donors are genetically deficient in PD-L1 or when PD-L1 blocking antibodies are administered [373]. In an MHC class II-mismatched murine skin graft model, PD-L1 blockade accelerated rejection with enhanced differentiation of CD4 Th1 alloreactive clones and reduced apoptosis of alloantigen-specific T lymphocytes [374]. Tolerance to skin allografts between B6 and BALB/c mice (MST >150 days) after induction of mixed allogeneic chimerism by donor BMT, sirolimus and anti-CD40L was not broken by coadministration of anti-PDL1 at the time of BMT: this is in contrast to experiments where tolerance to cardiac allografts induced using CTLA4-Ig was broken by administration of anti-PDL1 sixty days post-transplant [371]. This suggests a primary role for PD-L1 in inhibiting peripheral alloreactive clones.
In keeping with these findings, the PD-1:PD-L1 interaction has a role in the persistence of chronic viral infections such as hepatitis, HIV and lymphocytic choriomeningitis virus. PD-1 expression is increased on exhausted virus-specific CD8 cells, with PD-L1:PD-1 blockade increasing viral clearance even in the absence of CD4 help [375-380]. PD-1 has also been found to be upregulated on HIV-specific CD8 T cells in an untreated human population, correlating positively with functional impairment and reduced proliferative capacity, disease progression, and viral load: expression on CD4 T cells in the same population correlates negatively with CD4 count [381, 382]. Again, blockade improves both helper and cytotoxic T cell function [381, 383]. Functionally exhausted HIV CD8 T cells have been demonstrated with a PD-1⁺ CD160⁺ phenotype, with transcriptional upregulation of survival and functional inhibitors and downregulation of NFκB [384]. Furthermore, the presence of HIV-specific IL-10+ suppressor CD8 T cells is associated with increased expression of PD-1 on CD107⁺ CD8 T cells [385].
observations point to a role for PD-L1 in dampening the immune response to infection and inducing functional suppression of reactive CD8 T cells.

It appears that cross-talk between activated T cells and hepatocytes via PD-1:PD-L1 plays a role in modulating T cell responses. In a murine model of hepatitis B, HBV-specific CTLs were able to immediately secrete IFN-γ upon exposure to antigen in the liver but this diminished over 3 – 5 days in association with upregulation of PD-1 on CD8 T cells [386]. Blockade of PD-L1 is associated with an increase in IFN-γ+ CD8 T cells [378]. Viral infection and subsequently activated T cells have been shown to increase PD-L1 expression on hepatocytes, which can be further enhanced by the application of IFN-α or IFN-γ [387]. This IFN-γ mediated upregulation of PD-L1 on hepatocytes appears to be transient [378]. PD-L1 expressed on hepatocytes in response to IFN-γ secreted by activated T cells interacts with PD-1 on CD8 T cells to induce apoptosis, pointing to a mechanism whereby infected hepatocytes are able to dampen antigen-specific cytotoxic responses and avoid destruction [387].

1.8.4 The ‘liver tolerance effect’

The ‘liver tolerance effect’ refers to the unique capacity of the liver to induce antigen-specific tolerance. Mice, pig and rat studies of liver transplantation have shown that spontaneous allograft acceptance across a full MHC mismatch is possible [388-396]. Tolerant liver allograft recipients have been shown to accept subsequent skin and heart transplants from a strain-identical donor, as well as simultaneous heterotopic heart transplants [392-394, 396]. Expression of extra-hepatic antigens in the liver elicits a population of Tregs that suppress the immune response to those antigens,
perhaps facilitating the phenomenon of tolerance to a non-liver graft in simultaneous liver/non-liver transplants [397]. In fact, liver transplant from the same donor can even reverse rejection of heart, pancreas and skin transplants in some cases, suggesting that this tolerising effect holds even in the primed host and possibly in the face of a memory response [398-400]. This has significant implications for human populations which display high frequencies of alloreactive memory T cells pre-transplant [212]. In the clinical setting, some liver allograft recipients can be successfully weaned off immunosuppression [401-403]. Furthermore, simultaneous liver and renal or lung transplantation can prevent acute rejection and prolong graft survival in the non-liver graft, suggesting a role for the liver in developing donor strain-specific tolerance [404-407]. The ‘liver tolerance effect’ it is also thought to underlie the process of oral tolerance, where ingestion of peptides can leads to tolerance of these antigens – bypassing the liver with a shunt procedure abrogates this phenomenon [408, 409]. Another example of the liver’s propensity to tolerance is the persistence of viral hepatic infections (due in part to PD-L1 upregulation on hepatocytes).

The liver is constantly assaulted with an array of bacterial toxins and antigens from the gut and systemic circulation, yet it tends to secrete cytokines and activate pathways that promote tolerance rather than a destructive adaptive immune response. Various immune mechanisms as well as anatomic and histological factors have been postulated to confer the ‘liver tolerance effect’ [410].

From an anatomical perspective, the significant parenchymal mass of the liver has been postulated to act as a ‘cytokine sink’, diluting the cytokines necessary for T cell survival and inducing death by neglect [411]. It has also been offered as an explanation
for the finding that in rat studies where two kidneys and two hearts were transplanted to a single recipient, there was prolonged graft survival [412]. Furthermore, the fenestration of liver sinusoids allows direct contact between liver parenchymal cells and lymphocytes, which could facilitate interactions between naïve T cells and hepatocytes [413]. As hepatocytes are not professional APCs, they cannot fully activate these naïve cells and this may contribute to their subsequent death or induce anergy. While the liver has been revealed to be a site of naïve CD8 T cell activation, it produces CD8 T cells with defective cytotoxic function [414-417].

Resident liver sinusoidal epithelial cells (LSEC), dendritic cells (DC), Kupffer cells (KC) and hepatic stellate cells (HSC) can all present antigen and are thought to partly confer the unique tolerogenic properties of the liver [418]. These cells express low levels of MHC class II and costimulators and secrete IL-10, polarising the response towards a Th2 phenotype and stimulating the production of CD4⁺CD25⁺Foxp3⁺ regulatory T cells [419-421]. Liver sinusoidal epithelial cells are able to uptake soluble exogenous peptide through receptor-mediated endocytosis [422]. Cross-presentation of exogenous antigen to CD8 T cells via MHC class I elicits antigen-specific tolerance, whilst activation of naïve CD4 T cells via MHC class II directs helper T cells towards at Th0 phenotype [423, 424]. The presence of LSECs has been shown to be critical in suppressing alloreactive lymphocytes across MHC barriers [425]. Induction of CD8 unresponsiveness has been demonstrated to be mediated by interactions between PD-L1 on CD80/86low LSEC with PD-1 on cytotoxic T cells [426]. Contact between dendritic cells and LSECs reduces expression of CD80/86 on DCs and abrogates their ability to fully activate naïve CD8 T cells [427]. Kupffer cells, the resident macrophages of the liver, have been described as ‘tolerogenic APCs’ capable of producing
prostaglandins which inhibit DC-dependent T cell activation [428]. Models of concanavalin A mediated hepatitis suggest that IL-10 expression by Tregs and Kupffer cells is mandatory to confer tolerance [429].

Evidence is accumulating for the role of HSCs in influencing the formation of iTregs in the context of a bystander role or perhaps by acting as primary APCs [430-432]. Hepatic stellate cells are able to secrete TGF-β and store retinoic acid, both of which are known to encourage production of regulatory T cells, and their anatomical position in the space of Disse allows them to interact with LSECs, Kupffer cells and dendritic cells [433]. Retinoic acid causes differentiation of naïve T cells into Tregs in a TGF-β-dependent fashion, identifying it as a key driver in the regulating the immune response towards a suppressive phenotype [434]. TGF-β and all-trans retinoic acid (ATRA) have been shown to polarise naïve T helper cells towards a regulatory phenotype in mucosa and skin after antigen exposure [435-438]. Similarly, it has been shown that naïve hepatic CD4 cells encountering antigen in the context of activated HSCs, dendrocytes and TBG-β1 are polarised towards the Treg phenotype dependent on retinoid metabolism by HSCs [430]. HSCs have also been shown to mediate T cell apoptosis in association with upregulation of PD-L1 [439].

Hepatocytes engulf and degrade autoreactive cytotoxic T cells via ‘suicidal emperipolesis’, blockade of which results in autoimmune hepatitis [440]. During infection, hepatocytes can express aberrant MHC class II and direct naïve CD4 cells towards a Th2 phenotype while suppressing IFN-γ secretion from Th1 cells [387, 441, 442].
Spontaneous tolerance to liver transplants is dependent on donor passenger leukocytes (which for other solid organ transplants act as instigators of the immune response) as well as the establishment of a rapid immune reaction in recipient lymphoid tissues [443, 444]. It is hypothesised that donor passenger leukocytes may establish microchimerism, then instigate a limited graft-versus host reaction in recipient tissues [445, 446]. Activation of reactive clones followed by apoptosis leads to clonal deletion and plays a role in tolerance induction. The ‘activation-associated’ model proposes that such tolerance is conferred by passenger leukocytes [412, 447]. There is evidence that after transplantation, these APCs migrate to recipient lymphoid tissues whereupon host leukocytes transiently express high levels of IL-2 and IFN-γ, leading to exhaustive differentiation and activation-induced cell death (AICD) of alloreactive donor T cell clones [388, 444, 447-449]. The ‘high-dose tolerance’ model is based upon the observation that high-doses of antigen have been demonstrated to lead to rapid activation and elimination of reactive T cells, sometimes termed ‘tolerance by exhaustion’ [450, 451]. This has been demonstrated in animal models of experimental allergic encephalomyelitis, where high doses of antigen conferred activation of T cells and production of IL-2, with a subsequent deletion of these reactive cells [452].

While complex and not completely understood, the ‘liver tolerance effect’ holds great promise for the induction of tolerance to solid organ transplants, specifically via the use of liver-specific viral vectors to induce hepatic tolerance to donor antigens.
‘An inefficient virus kills its host. A clever virus stays with it’ - James Lovelock

Gene therapy holds promise for correcting host pathology through manipulation of DNA expression. Gene therapy can involve transfer, repair or silencing. Transfer of genetic material using viral vectors enables the expression of a particular functional protein within the host. Genetic repair attempts to correct a flaw in host DNA, while silencing switches off production of a pathogenic protein. There has been particular interest in gene transfer for correcting monogenic disorders such as severe combined immunodeficiency and Haemophilia B, with a number of human trials already performed [453-456]. There is also much interest in gene transfer therapy in solid organ transplantation for the induction of transplant tolerance through modulation of co-stimulatory pathways, manipulation of cytokine expression and apoptosis pathways, immunomodulation via enzyme expression, leukocyte migration, and transfer of donor MHC or transduced APCs and/or lymphocytes (reviewed in [457]).

The transfer of genetic material can be accomplished in vivo through local or systemic inoculation or ex vivo where the target of interest is collected and modified outside of the organism before return to the host. Transfer of synthetic DNA can be accomplished by transfection or transduction. Transfection involves transfer of DNA via physical, chemical or electrical methods [458, 459]. High transfection efficiency can be accomplished by synthetic delivery systems which usually exploit polycationic polymers and/or cationic lipids complexed with nucleic acid (polyplex, lipoplex or
lipopolyplex systems) or lipid encapsidation (liposomes) [460]. Alternatively, transduction utilises recombinant virus as a vector for gene transfer. Entry of these vectors is mediated by cell-surface receptors and is generally more efficient than synthetic systems at delivery to target cells – this is particularly true of recombinant viral vectors [461-463]. The characteristics of an ideal vector for gene transfer are shown in Table 6 (based on [462]). Recent advances in viral vector technology, ongoing evidence of successful liver-targeted therapy in inducing high-level stable transgene expression, and the unique ability of the liver to induce tolerance to foreign antigens has spurred interest in utilising viral vector therapy as a pathway to transplantation tolerance.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue tropism</td>
<td>Maximise therapeutic potential via targeted delivery</td>
</tr>
<tr>
<td>Vector capacity</td>
<td>Size limitation imposed on transgene</td>
</tr>
<tr>
<td>Continued expression in dividing cells</td>
<td>Though genomic integration or episomal replication</td>
</tr>
<tr>
<td>Targets quiescent cells</td>
<td>Can become incorporated in resting cells</td>
</tr>
<tr>
<td>Site-specific integration</td>
<td>Reduce risk of insertional mutagenesis</td>
</tr>
<tr>
<td>Easy to produce</td>
<td>High-titre preparations made easily and cheaply</td>
</tr>
<tr>
<td>Appropriate transgene expression</td>
<td>Sustained or regulated (depending on application)</td>
</tr>
</tbody>
</table>

Table 6: Characteristics of the ideal vector
1.9.1 **Adeno-associated virus (AAV)**

One of the most well-studied and best understood of the viral vector candidates is the human adeno-associated virus (AAV). The human adeno-associated virus (AAV) was isolated in 1965 as a contaminant of adenovirus preparations [464]. It is a *Dependovirus* of the *Parvoviridae* family and is a replication-defective, non-enveloped virus of approximately 20 nanometers length [465]. AAV requires a helper virus for proliferation, normally provided by adenovirus or herpes virus [466]. Its unique features make it particularly attractive for use as a viral vector. AAV is non-pathogenic and initiates at most a mild immune response [467]. It can infect both dividing and quiescent cells and may persist in an extrachromosomal state or integrate stably into a specific locus of human genome, minimising the risk of insertional mutagenesis which can be seen with other viruses (such as retroviruses) [468-470].

Structurally, it is composed of linear single-stranded DNA of approximately 4,680 bases which codes for the two open reading frames *rep* (responsible for gene expression and replication) and *cap* (encoding structural proteins) flanked by 145-nucleotide inverted terminal repeat sequences (ITRs) (see Figure 10) [471, 472]. The ITRs are required *in cis* and are necessary for efficient viral replication: they are folded into hairpin-shaped structures which contribute to the ability of AAV to undergo primase-independent transcription of a second DNA strand [473, 474]. A *Rep* binding element (RBE) tetranucleotide repeat sequence (GAGCGAGCGAGC) within the ITR allows binding of *rep* proteins with endonuclease activity and subsequent replication [475]. ITRs also play a role in genomic integration and encapsidation, as well as possibly assisting in replication within episomal concatamers while conferring resistance to their degradation [476-479].
The rep gene contains three promoters - p5, p19 and p40 - which can facilitate transcription and lead to the production of multiple gene products from overlapping reading frames (ORF) by splicing of introns. The p5 and p19 promoters upstream of the rep ORF can produce two proteins, each with the ability to splice an intron, giving rise to the four protein products Rep40, Rep52, Rep68 and Rep78 (where the number denotes their length in kiloDaltons) [480]. Rep68 and Rep78 bind RBE sequences and mediate site-specific integration through GCTC repeating motifs: in humans this occurs in chromosome 19 at the position termed AAVS1 [468, 478]. Approximately 0.1-0.5% of infectious wild-type AAV particle integrate into the host genome at this site [481].

The p40 promoter facilitates transcription of the cap proteins, termed VP1, VP2 and VP3 [482]. Sixty of these are arranged in the ratio 1:1:10 to form the mature capsid [483]. More than ten AAV serotypes (AAV1-11) exist which exhibit no immune cross-reactivity and differ in either their capsid proteins or genomic structure [484]. The serotype of the virus imparts its tissue tropism, immunogenicity and propensity for...
infection and integration. The tissue tropism of the different serotypes is shown in

**Table 7** [484-499].

<table>
<thead>
<tr>
<th>Tissue Tropism</th>
<th>Serotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>AAV8,9</td>
</tr>
<tr>
<td>Kidney</td>
<td>AAV2</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>AAV1,6,7,8,9</td>
</tr>
<tr>
<td>Central nervous system</td>
<td>AAV1,4,5</td>
</tr>
<tr>
<td>Retinal pigment epithelium</td>
<td>AAV4,5</td>
</tr>
<tr>
<td>Photoreceptor cells</td>
<td>AAV5</td>
</tr>
<tr>
<td>Lung</td>
<td>AAV9</td>
</tr>
<tr>
<td>Heart</td>
<td>AAV8</td>
</tr>
</tbody>
</table>

**Table 7: Tissue tropism of AAV serotypes**

When infected with AAV, virus particles bind to glycosaminoglycan receptors and enter the host cell. The steps involved in infection include cell-receptor binding, endocytosis, nuclear transport, uncoating and conversion of ssDNA to a double-stranded DNA (dsDNA) transcription template (see **Figure 11**) [500]. The specific receptor bound is dependent on the serotype: for example AAV2 attaches to the heparan sulphate receptor [501, 502]. Interaction with the receptor and various co-receptors leads to endocytosis of the virus particle. In the case of AAV2, co-receptors include fibroblast growth factor receptor-1, hepatocyte growth factor receptor and
integrin $\alpha_\text{V}\beta_5$ [503-505]. Processing of the virions within the endosome influences transduction, with resultant release and perinuclear accumulation [506]. The exact mechanisms surrounding uncoating and nuclear penetration are not entirely clear [484]. The ssDNA sequence is converted to dsDNA by annealing of single strands or host-cell polymerase [500]. The majority of the resulting dsDNA persists in the nucleus as episomal concatamers formed largely by intermolecular recombination of circular genomes, while the remainder undergo random genomic insertion [507-512]. In the absence of a helper virus, AAV can persist in a latent state, making it particularly attractive for inducing the long-term expression of gene products [513, 514]. AAV capsid proteins may undergo ubiquitination and hence be directed towards proteasomes, generating peptides which for display on MHC class I molecules and priming the immune system against capsid antigens.

Figure 11: Integration and expression of rAAV
Barriers cited to clinical applicability include the risk of insertional mutagenesis or germline transmission and restriction imparted by the length of the transgene cassette [515]. Liver tumours have been noted in mice injected with AAV as neonates, but studies in mature animals and humans have not shown a link between rAAV administration and oncogenesis [516, 517]. Rabbit studies have shown that systemic administration of AAV2 vectors results in vector DNA in semen, but that this was lost in a time and dose-dependent fashion [518]. Furthermore, although rAAV does have limited capacity, vector genome linkage has enabled splitting of large genes between two vectors, effectively doubling the coding capacity of AAV2 [519-521].

1.9.2 RECOMBINANT ADENO-ASSOCIATED VIRAL (RAAV) VECTOR DESIGN

The unique properties of the adeno-associated virus, namely its lack of pathogenicity, ability to infect dividing and quiescent cells, need for helper virus to facilitate infection, and elegant genome make it attractive as a recombinant viral vector for gene transfer therapy. Removal of the open reading frames and insertion of a promoter and transgene product into the expression cassette facilitate transfer of the desired gene while rendering the vector unable to replicate and abrogating the ability for site-specific genomic integration (which is dependent on rep proteins, see 1.9.1 Adeno-associated virus (AAV)). Robust gene expression from rAAV vectors occurs even in the absence of genomic integration [509]. ITRs may contribute to the persistence of the virus by assisting in the formation of episomal concatamers, conferring resistance to their degradation and assisting with second strand replication [522].
Choice of serotype and production of hybrid rAAV play critical roles in maximising the efficiency of the vector. Hybrids are formed through transcapsidation, peptide adsorption onto the capsid surface and construction of chimeric or mosaic capsids [500]. Hybrid vectors often employ the AAV2 genome (which is the best characterised), combined with capsid proteins of a different serotype that maximise kinetics of expression and appropriate tissue tropism. The use of the serotype 8 capsid in murine models imparts tropism to hepatic tissues and rapid uncoating of vector genomes, which can be augmented by use of liver-specific promoters to optimise hepatic transgene expression [467, 476, 495]. In fact, the serotype 8 capsid has proven to confer impressive liver tropism in mice, rats and non-human primates with rapid kinetics of expression [476, 495, 523-526]. The AAV2/8 hybrid with a hAAT (human α-1 antitrypsin) promoter and WPRE (woodchuck hepatitis virus post-transcriptional regulatory element) can efficiently confer stable, long-term transgene hepatic expression (see Figure 12) [467, 527, 528]

Figure 12: Mosaic capsid recombinant adeno-associated virus
1.9.3 RAAV IN ANIMAL STUDIES

Liver-directed rAAV therapy has been shown to induce tolerance to a range of transgene products such as human α-1 antitrypsin, α-galactosidase, β-galactosidase, Factor IX and chicken ovalbumin [529-535]. Murine studies have shown that targeting AAV to the liver can abrogate the formation of antibodies seen when the same transgene was given intramuscularly [536-538]. Remission of type 1 diabetes was seen in a rat model which utilised rAAV to induce a single-chain insulin analogue (SIA) in the liver under the control of hepatocyte-specific L-type pyruvate kinase promoter, which was tolerated without any histological evidence of damage to hepatocytes, rise in transaminases, or production of anti-SIA antibodies [539]. Animal models of tolerance induction using hepatic transgene expression have been promising with evidence for CD4 anergy and increased pools of Tregs which mediate suppression of antigen-specific CD8 T cells [531-535]. Increased Treg populations after hepatic transduction have also been shown to suppress the formation of antibodies to the gene product [540].

Hepatocyte expression of the immune modulators indoleamine 2,3-dioxygenase, CTLA4-Ig and vIL-10 using rAAV in order to achieve tolerance have been studied. Expression of indoleamine dioxygenase in liver allografts using rAAV2/8 resulted in strong protein expression in donor liver and a reduction of 50% in serum tryptophan levels but no prolongation of graft survival [525]. Studies utilising costimulatory blockade between B7 and CD28 have been more promising. Recombinant AAV expressing CLTA4-Ig has been used to transduce hepatocytes of transplanted livers between DA and LEW rats and shown an increase in graft survival from 9 to 109 days [541]. Using the same genetic strain, it has been shown that using rAAV-hCTLA4-Ig
transduction of recipient hepatocytes and low-dose FK506 administration in orthotopic liver allografts, long-term graft survival can be achieved [542]. A similar model, again utilising rAAV-CTLA4-Ig, increased cardiac allograft survival from 6 to 64 days, with concurrent administration of anti-ICOS (inducible co-stimulator) antibody resulting in long-term graft acceptance (>300 days) [543]. This was found to be due to increased proportions of CD4+CD25+ Tregs in tolerance recipients; however subjects were not able to accept skin grafts from a same-strain donor. The well-characterised DA to LEW model has also been employed to study the effects of viral IL-10 using AAV-mediated expression in recipient hepatocytes, with a marginal increase in cardiac allograft survival noted [544].

1.9.4 rAAV IN CLINICAL TRIALS

The use of rAAV therapy in humans has been targeted towards the treatment of diseases resulting from loss of function of a single protein product. The most widely studied of these is the correction of Haemophilia B using liver-targeted rAAV to induce hepatic expression of a functional FIX protein. Following the success in animal models, a human clinical trial of rAAV2 expressing human FIX injected into the hepatic artery of seven Haemophilia B patients showed no acute toxicity and therapeutic FIX levels at the highest vector dose (2 x 10^{12} vg/c/kg) [455]. Subsequent expression lasted approximately 8 weeks before destruction of transduced hepatocytes. Loss of transgene expression with a transient transaminitis was noted, mediated by CD8 T cells primed by MHC class I molecules displaying capsid proteins [455, 545].
More recently, an attempt to optimise expression and minimise antigenicity has been made, with eight subjects receiving a self-complimentary, codon-optimised human FIX transgene packaged in AAV2/8 [454]. The result has been long-term expression of human FIX (followed for a period of 3 months to 2.5 years at time of publication): five patients were able to discontinue FIX therapy and the other three were able to reduce time between doses [454, 546]. Three of the patients had a rise in transaminase levels, one due to an anti-AAV8 capsid response, all of which normalised with a short course of glucocorticoid therapy without loss of FIX expression [454]. Manipulation of vector design holds promise for inducing long term hepatic expression of transgenes and via this harnessing the liver tolerance effect in a step towards operational tolerance. The safety of rAAV in human subjects has been exemplified in promising trials aimed at Leber’s congenital amaurosis and Parkinson’s disease [547-550]. The fact that liver-targeted rAAV has already been used successfully and safely in human trials indicates that this technology is rapidly gaining ground.

1.9.5 IMMUNE RESPONSE TO RAAV

Determinants of the immune response to rAAV include the route of administration and site of expression, the vector design and dosage, level of transgene expression, genetic background of the recipient and pre-existing memory responses [467, 523, 531, 533, 535, 551, 552]. Cellular responses to capsid antigens occurring after repeated vector administration using AAV1 and AAV2 were first noted with intramuscular administration of AAV vectors [553-556]. Capsid proteins processed and displayed in association with MHC class I have been shown to facilitate destruction of
transduced hepatocytes [545]. Pre-existing memory responses against AAV2 capsid proteins in humans have been shown to confer cytotoxicity to AAV1 and AAV8 serotypes, possibly through nucleotide homology [486, 555]. A recent study in human subjects showed that pre-existing NABs and AAV-capsid reactive CD8 T cells specific for AAV1 were not correlated, suggesting a role in screening patients for both NABs and cellular responses prior to vector administration [557].

While transducing the liver tends to promote tolerance to the product, targeting other tissues such as skeletal muscle tends towards immunogenicity. Canine and murine studies of Factor IX expression have revealed higher expression of the transgene and lack of formation of neutralising antibodies when rAAV is targeted to the liver compared to the muscle [536-538, 558, 559]. A study of rAAV delivered by peripheral vein infusion containing a hybrid liver-specific promoter and expressing hu-FVIII showed high levels of expression in both mice and macaques, with the formation of neutralising antibodies that could be eliminated with concurrent doses of immunosuppression [560].

Transgenic mouse studies have shown that muscle-targeted expression of a rAAV transgene product can be immunogenic in one strain but lead to no response in a different strain, suggesting an important role for the genetic background insofar as it determines the ability for different MHC alleles to display particular peptides [552]. Furthermore, replacement transgene products which were not present during the development of self-tolerance in the host are more likely to induce an immune response than those which were present but expressed at low levels or with only minor variation of the protein sequence [552, 561].
In terms of vector design, the promoter plays a role in the subsequent immune response. Murine models employing the cytomegalovirus-immediate early (CMV-IE) promoter yielded lower human factor IX (hu-FIX) expression in hepatocytes and the production of anti-hu-FIX antibodies. When the CMV-IE promoter was replaced with the human elongation factor promoter, higher levels of expression and no immunological response was seen [562]. The use of liver-specific promoters can assist with tissue targeting, transduction and maximising transgene expression. Achieving high-levels of expression in the liver may help to confer tolerance by inducing exhaustive clonal differentiation of reactive cell populations [447].

Route of administration also plays a role in determining the immune response. Routes of rAAV transfer to the liver include portal vein, splenic and intraperitoneal inoculation [467, 476]. Mice models of AAV2 targeted to hepatocytes revealed a B cell response to capsid proteins which was found to be T cell dependent or partially T cell independent, depending on whether the vector was administered into the tail or portal vein [563]. Intraperitoneal cavity injection of rAAV2/8 is shown to confer stable high-level hepatic expression in mice [476]. Comparison of rAAV-hCTLA4lg (human cytotoxic T-lymphocyte-associated antigen 4-immunoglobulin) administered through ex-vivo portal vein infusion, intravenous and intramuscular injection to orthotrophic rat liver transplant recipients showed higher titres from portal vein infusion than the other modalities [564].

A recent study of donor MHC class I K\(^b\) transfer with rAAV in a murine model of skin allografts revealed that at low doses (5 \(\times\) 10\(^6\) vector genome copies), the immune system was primed and an accelerated rate of graft rejection was seen, while at high
doses (5 x 10^{10} vector genome copies), tolerance ensued [523]. An rAAV1 expressing CTLA4-Ig targeted to muscle was found to potentiate the formation of antibodies at doses of 10^9 vgc, while doses of 10^{12-13} vgc conferred long-term tolerance [565]. While the formation of antibodies to capsid proteins can render repeated vector administration ineffective in transducing target cells, using low vector doses has been shown to reduce the formation of NABs [566]. Similarly, a study of rAAV2/8 therapy for haemophilia B in human subjects revealed greater capsid-specific T cell responses with higher doses of vector [546]. Therefore, optimal vector dosage requires a balance between achieving tolerance to the product and minimising the immune response to immunogenic capsid proteins, underscoring the importance of transduction efficiency.

Humans have almost ubiquitous exposure to wild-type AAV and the initial immune response is principally humoral through the formation of neutralising antibodies to capsid proteins: a Th2 response with a rise in IL-10 and lymphocytosis can be seen after rAAV administration [567-569]. Serum neutralising antibodies (NAB) to serotypes AAV 1 2, 3 and 5 are the most common [561]. Low levels of circulating NABs have been shown to reduce the efficiency of hepatic transduction with rAAV2 vector in murine models [563, 570, 571]. Transfer to human therapy should consider the immune response to AAV from pre-formed antibodies and the possibility of a cytotoxic responses to capsid proteins [555, 557, 561, 567, 572, 573]. Adoptive transfer studies of capsid-specific CD8 T cells to syngeneic mice which had received liver-targeted rAAV failed to show destruction of AAV-transduced hepatocytes, suggesting there may be disparities in hepatocyte cross-presentation of capsid antigens in human and animal models [574]. The underlying mechanisms explaining these differences have not yet been elucidated.
1.9.6 ADJUVANTS TO AAV THERAPY

As exemplified in human trials of haemophilia B, discordance in expected responses between mice and humans can be observed [455]. High rates of NABs to certain AAV serotypes in the human population demands careful vector construction and close monitoring of test subjects. Transcapsidation or naked AAV-plasmid DNA packaging in liposome complexes may provide avenues to avoid pre-existing immunity to AAV capsid serotypes [575, 576]. It has been suggested that some level of immunosuppression concurrent with vector administration may be needed to minimise the immune response to the vector and facilitate efficient transduction and subsequent tolerance [455]. This approach has been utilised in both animal and human models with success [454, 542, 560]. Recently discovered Tregitopes have received interest due to their ability to induce Tregs and suppress immune responses [360, 577]. There may be a future role in harnessing their desirable properties in conjunction with rAAV therapy to induce tolerance.

It has become increasingly clear that endosomal processing pathways and nuclear trafficking are critical factors regulating the efficacy of transgene expression [578, 579]. Uptake into endosomes, activation of ubiquitin ligase and subsequent direction to proteasomes for degradation is considered to limit the efficiency of rAAV transduction and also facilitates capsid peptide display by MHC class I molecules. Animal studies of AAV administration to lung and liver with ubiquitin-ligase or proteasome inhibitors have shown increased levels of transduction as well as faster transgene expression [578, 580, 581]. The inhibition of ubiquitin-proteasome pathways also decreases capsid protein display on MHC class I which may limit the formation of cytotoxic responses and the formation of memory pools, allowing for
repeated vector administration [582]. Co-administration of such inhibitors may play a role in future human trials. Avoidance of ubiquitination has been incorporated into vector design by introducing point mutations to tyrosine residues in AAV2 capsid proteins, inhibiting their phosphorylation and preventing them from entering the ubiquitin-proteasome pathway [583-585].

1.9.7 DONOR MHC EXPRESSION USING RAAV IN TRANSPLANTATION

In most gene therapy studies, tolerance to the transgene product is highly desirable because it permits long-lasting expression of the gene product and a durable therapeutic effect. Extension of this systemic tolerance to a transplanted organ or tissue which expresses the same gene product endogenously is a logical progression. Rodent studies have explored the ability of hepatic expression of donor-MHC in recipients to overcome rejection to skin and solid organ transplants. Retrograde perfusion of Male Lewis rat livers with AAV expressing the Dark Agouti class I MHC soluble RT1.Aa antigen have shown long-term expression of the transgene, and subsequent heterotopic cardiac transplants from DA (RT1a) donors to the transfected LEW (RT1I) rats demonstrated a modest prolongation in allograft survival of 1 to 2 days [544].

Recent work in our laboratory with AAV2/8 has shown that expression of donor MHC in recipient livers can overcome naïve and memory alloresponses to skin grafts in a murine model using B10.BR and 178.3 mice mismatched at a single H2 class I locus [523]. B10.BR mice inoculated with 5 x 10^{10} vgc of AAV2/8 containing the K^b gene (rAAVKb) exhibited stable, high-level expression of H-2K^b with no evidence of liver
damage as assessed by serum transaminase levels and histological examination. Subsequent skin grafts from 178.3 mice to transduced B10.BR animals demonstrated operational tolerance in both naïve recipients and in those who had already rejected a primary graft [523]. ELISPOT assay revealed that administration of rAAVKb to primed or unprimed B10.BR reduced the population of CD8 T cells able to produce IFN-γ upon stimulation with 178.3 splenocytes. Des TCR T cells with transgenic TCRs recognising the Kb antigen proliferated after exposure to Kb on rAAVKb-transduced hepatocytes but were not deleted, suggesting the alloreactive cells are rendered incompetent or ‘functionally silenced’. Tolerance was specific to the transgene product; grafts from C57BL/6 mice (b-haplotype) were not accepted by mice which had been rendered tolerant to 178.3 grafts, revealing no evidence for epitope spreading, while transduction of B10.BR mouse livers with the third party antigen K\textsuperscript{d} did not prolong survival of Kb-bearing 178.3 skin grafts. These results are particularly promising due to the known difficulties in obtaining tolerance to skin grafts and overcoming memory responses, underscoring the potential for liver-directed AAV gene expression to induce operational tolerance.

1.10 AIMS

We have previously demonstrated that the rAAVKb vector can elicit stable, long-term expression of K\textsuperscript{b} molecules on recipient hepatocytes without inducing an inflammatory response [523, 586, 587]. Recent work in our laboratory has shown successful transduction of the K\textsuperscript{b} gene to recipient B10.BR murine liver using an rAAV2/8
(rAAVKb) vector can overcome naïve and memory responses to skin grafts from 178.3 (K^{k,b}) to B10.BR (K^b) mice, conferring operational tolerance.

The aims of this project are to confirm this finding and further define the pathways by which this tolerance is induced. Possible means of recognition of the K^b molecules include 1) direct recognition by recipient alloreactive CD8 T cells (the majority of this population being CD8 dependent); 2) presentation of K^b peptides on B10.BR recipient hepatocyte K-haplotype class I molecules (K^k, K^k, L^k) to CD8 T cells; 3) cross-presentation of K^b peptides on recipient Class I on APCs in the liver and systemically to CD8 T cells and/or 4) presentation of K^b on recipient class II K haplotype molecules (I{E}^k, A{I}^k) to CD4 T cells by recipient APC which may result in the generation of T regs

By eliminating direct recognition, it is possible to ascertain whether this is a requirement for tolerance induction via pathway 1. Construction of a mutant rAAVD227K vector with a point mutant in the acidic loop of the K^b molecule which abrogates CD8 co-receptor binding will largely eliminate the first pathway without an effect on pathways 2 - 4. The immune response to this defective donor MHC class I can be used to investigate whether direct antigen recognition of donor class I MHC is necessary to induce tolerance. Specifically, the role of direct antigen presentation will be explored by comparing skin transplant rejection profiles in mice inoculated with rAAVKb and versus mutant rAAVD227K.

Additionally, the importance of PD-L1:PD-1 interactions between hepatocytes and CD8 T cells in inducing tolerance is investigated. IFN-γ mediated upregulation of this ligand on hepatocytes and cytotoxic T cells followed by silencing of reactive T cell clones via PD-L1:PD-1 interactions has been shown associated immunosuppressive phenotype.
We explore the contribution of PD-L1 in CD8 T cell tolerance induction, by examining PD-L1 expression on hepatocytes after rAAVKb and rAAVD227K inoculation and the effect of PD-L1 blockade on skin graft survival.

Finally, evidence for Treg induction is investigated in naïve B10.BR mice primed by rejection of a 178.3 graft and subsequently given rAAVKb, as upregulation of regulatory T cell populations which suppress antigen-specific CD8 T cells has been demonstrated after liver rAAV gene transfer [532-534].
2.0 METHODS

2.1 SKIN GRAFTING

2.1.1 ENVIRONMENT

All procedures were performed in the Microsearch Microsurgery Facilities at the University of Sydney. The microsurgery room is a designated PC2 area, with complete animal surgical facilities (see Figure 13). The Leica M651 MSD microscope was used for operating, with pictures taken using the Leica DFC400 camera attachment (both from Leica Microsystems, NSW, Australia). Animals were housed and monitored in the Bosch Rodent Facility at the University of Sydney with care provided by the researchers and animal house staff. All experimental protocols are approved by the university’s animal care and ethics committee.

Figure 13: Microsurgery facilities
2.1.2 Donor

The donor animal provides skin for grafting onto recipient mice. The animal is anaesthetised by placing it in a bell jar lined with isoflurane-soaked gauze, then transported to the operating bench where anaesthesia is maintained with a mixture of 1-3% isoflurane (Baxter, Deerfield, IL) and 200-400cc/min of 100% oxygen delivered from an anaesthetic machine to a 20ml syringe with the plunger removed. The animal’s head is able to fit snugly into the syringe chamber to ensure it receives the anaesthetic. The tail is amputated at the base using a scalpel blade and the donor animal then killed by cervical dislocation.

A median incision is made down the length of the tail and the tail skin is removed proximally from the underlying subcutaneous tissue by blunt dissection, from which point it can be easily stripped from the tail. The tail skin is spread flat onto clean saline-soaked gauze and cut at 5mm intervals, producing approximately 6 squares of donor skin (approximately 5mm x 5mm) from each tail harvest. The skin is then wrapped in saline soaked gauze and rested on a bed of crushed ice until grafting occurs.

2.1.3 Recipient

The recipient animal is anaesthetised as previously described (see 2.1.2 Donor). It is positioned in a ventral position on the operating table with the head placed into the chamber of a 20ml syringe which delivers a mixture of 1-3% isoflurane (Baxter) and 200-400cc/min of 100% oxygen (the concentration being titrated against the animal’s respiratory rate during the procedure). The back of animal is sterilised with an 80%
alcohol solution and the upper back then shaved using a size 20 scalpel blade to provide a clean bed of skin. The skin is washed with 80% alcohol solution and then normal saline and the area patted dry with clean gauze.

An area of skin (approximately 5mm x 5mm) is dissected from the subcutaneous tissue under microscope-assisted vision from each side of the animal’s upper back. Any bleeding is controlled with pressure and/or hand-held diathermy. Once the area is prepared it is flushed with saline if necessary to remove any unwanted hair or contaminants which could compromise successful grafting onto the new bed. Each bare area is then grafted using the donor tail skin. The allogeneic graft is position on the right side and the syngenic control graft on the left side. Fixation is performed at 3 – 4 points at the edge of the graft using skin glue adhesive (see Figure 14). At this stage the anaesthesia is discontinued.

Figure 14: Newly grafted B10.BR mouse
A bandage is fashioned by cutting the adhesive off one side of a Band-Aid® (Johnson and Johnson, New Brunswick, NJ) and placing its padded area adjacent to that of a standard Band-Aid®. In this way, the non-adhesive cushioned area is doubled and can overlie both grafts. The adhesive edges of the bandage are then placed around the circumference of the animal, firm enough to provide a protective covering for the grafts while being mindful not to restrict breathing. Small segments are removed from the bandage near the upper limbs and neck to facilitate normal mobility. Antibiotic prophylaxis is administered as 10 mcg of ampicillin (Aspen, Durban, SA) and analgesia is provided with 2-3mcg of buprenorphine (American Regent, Shirley, NY), each injected intramuscularly into opposite thighs. Recipient animals are warmed on a heating pad set to 37°C and monitored until they regain full consciousness and normal mobility. Each grafted animal is housed separately and monitored closely as per the University of Sydney Ethics Committee Approval Protocols K00/8/-2009/4/5104 and K00/2-2013/3/5946.

2.1.4 ANIMAL MONITORING

Daily monitoring was required for the first 10 days post-operatively, with a minimum of 3 days per week monitoring thereafter. Bandages are removed at 7 days after grafting to facilitate monitoring for rejection. Rejection was defined as >80% loss of graft viability, seen macroscopically as dryness, elevation and darkening of the graft which subsequently detached, leaving an open wound.
2.2 **Viral Vectors**

Two adeno-associated viral vectors were used in this thesis. Both vectors are AAV2/8 hybrids and incorporate the hAAT liver-specific promoter and human Apolipoprotein E (ApoE) enhancer, a combination which is known to confer hepatocyte specificity and high-levels of transgene expression. The first (rAAVKb) encodes the murine MHC class I K\(^b\) antigen. The second (rAAVD227K) vector expresses a mutant K\(^b\) with a single amino acid substitution at position 227 in the K\(^b\) α chain which abrogates CD8 co-receptor binding (see [1.3.3 Site directed mutagenesis of MHC class I abrogates direct antigen recognition by CD8 T cells](#)).

### 2.2.1 rAAVKb Vector

The pAM2AA_Kb plasmid was donated by Szun Tay, David Bowen and Patrick Bertolino of the Centenary Institute, Sydney, Australia. In brief, the H-2K\(^b\) coding sequence was amplified from C57BL/6 cDNA with an optimised Kozak sequence (forward primer 5’ CGG AAT TCG CCA TGG TAC GGT GCA 3’, reverse primer 5’ CGC AAG CTT CAC GCT AGA 3’) [523]. A pAM2AA backbone incorporating the liver specific hAAT promoter and human ApoE enhancer with AAV2 ITRs was used for vector insertion.

### 2.2.2 rAAVD227K Vector

To create the rAAVD227K vector, the pAM2AA_Kb vector was first sequenced so that the nucleotides corresponding to position 227 of the K\(^b\) molecule could be identified for site-directed mutagenesis. The pAM2AA vector backbone consists of a multiple
cloning site (MCS) incorporating several restriction enzymes flanked by the human α-1 antitrypsin promoter and a downstream woodchuck hepatitis virus post-transcriptional regulatory element (see Figure 15).

Figure 15: The empty pAM2AA vector

Sequencing of pAM2AA_Kb was performed using the hAAT (5’ GATCCCAGCCAGTGGACTTA 3’) and WPRE primers (5’ ACTGTGTTTGCTGACGCAAC 3’). Additional forward (5’ CTCTGGCTGTGAAGTGGGG 3’) and reverse primers (5’ GGATCAGCTCCTCCCCATTC 3’) were necessary to complete sequencing of the entire K\textsuperscript{b} insert and produced approximately 400 base pairs of overlap (for details of the primers and K\textsuperscript{b} insert sequence refer to Appendix). The sequence was submitted to the UniProt Knowledgebase Resource (http://uniprot.org) for annotation. Once sequencing was performed, the start of the mature protein was identified and position 227 identified (as given in [588, 589]). The codon for residue 227 was identified as GAC (aspartic acid), which was mutated to AAG (lysine). Comparison of the native K\textsuperscript{b} and mutated D227K with multiple sequence alignment using ClustalW from ExPASy SIB
Bioinformatics Resource Portal (http://www.expasy.org/) confirmed the presence of the mutation at position 227, with conserved homology of the remaining sequence. The sequence of the mutant D227K K<sup>b</sup> DNA was sent to Life Technologies™ for codon optimisation and synthesis. The cDNA was supplied cloned into a pMA-T backbone (pMA-T_D227K) (see Figure 16).

![Plasmid Map:](image)

Figure 16: K<sup>b</sup>-D227K within the pMA-T backbone
(courtesy of Life Technologies™)

Competent Escherichia coli (E. coli) DH5α cells were transformed with pMA-T_D227K and expanded in culture. A 1μL sample was digested with EcoR1 and HindIII to release the insert for the gene of interest (D227K). The products were run on a 1% agarose gel and the band containing the D227K for the insert was extracted from the gel and purified using the Wizard® SV Gel and PCR Clean-up System (Promega, Madison, WI). D227K was subcloned into the pAM2AA backbone using T4 DNA ligase to create the
pAM2AA_D227K plasmid. The pAM2AA_D227K sequence was verified and the plasmid grown in *E. coli* SURE2 (stop unwanted rearrangement events) cells prior to purification and packaging.

### 2.2.3 VECTOR PACKAGING AND PURIFICATION

The rAAVKb and rAAVD227K vectors were produced by triple transient transfection of HEK 293 cells using calcium phosphate. The plasmids used were pXX6, p5E18VD2/8 (donated by Ian Alexander of Children’s Medical Research Institute and the Children’s Hospital, Westmead, Sydney), and pAM2AA_Kb (donated by Szun Tay, David Bowen and Patrick Bertolinno of the Centenary Institute, Sydney, Australia) or pAM2AA_D227K.

After culturing the transfected cells for 48 hours they are lysed by repeated freeze-thaw cycles. Cesium chloride gradient purification was then utilised to separate the virions from the cell lysate which is distributed into six 15ml Falcon tubes. Ten fractions were collected from each tube and the fractions containing the virus identified by qPCR, pooled and dialysed against three changes of 4L of PBS (Sigma, St. Louis, MO) with 1.97ml of 1M MgCl₂ (Sigma) and 1.8ml of 2M CaCl₂ (UNIVAR Redmond, WA) for 5 hours each. Subsequently, the virions were concentrated by centrifugation at 4000g at 4°C using the Vivaspin®20 centrifugal concentrator (Vivaproducts, Littleton, MA). The final product was analysed by real-time PCR to estimate the viral genome copies (vgc)/ml. The samples were subsequently distributed into $5 \times 10^{10}$ or $5 \times 10^{11}$ vgc aliquots into autoclaved eppendorfs and stored at -80°C.
Prior to injection, the samples were thawed and made up to 500μL in sterile phosphate buffered saline (PBS).

2.3 RAAVKb/RAAVD227K INOCULATION

Inoculation of murine recipients with recombinant adeno-associated viral vector was accomplished through penile vein injection. The animal was anaesthetised in a bell jar lined with isoflourane-soaked tissue and then transported to the operating table where anaesthesia was maintained with a mixture of 1-3% isoflurane (Baxter) and 100% oxygen titrated to respiratory rate. The foreskin was retracted and the penis cleaned with an 80% alcohol solution. The vector dose is drawn up into a 1ml insulin syringe with a 29 gauge needle, being careful to expel any air to prevent embolism. Under microscope-assisted vision, the needle is introduced into the dorsal penile vein and the entire dose of vector administered through slow intravenous injection. The foreskin is then brought back over the penis to prevent paraphimosis. Anaesthesia was then discontinued and the animal allowed to fully recover before being transported back to the animal house for monitoring.

2.4 ANTI-PDL1/CONTROL ANTIBODY INOCULATION

The anti-PDL1 antibody and control antibodies (see Table 8) were given via intraperitoneal injection. For the initial injection 500mcg of antibody were injected (500μL), while for additional doses 250mcg are mixed with sterile PBS to a total volume of 500μL. The animal is briefly anaesthetised by placing it in a bell jar lined
with isoflurane-soaked gauze. Then animal is then held from the dorsal surface so that the abdomen is exposed and the skin drawn taut (see Figure 17).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Target</th>
<th>Concentration</th>
<th>Clone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-mouse CD274</td>
<td>Programmed Cell Death Ligand 1</td>
<td>0.5mg/ml</td>
<td>Clone: MIH5 Lot: E17964-101 Cat: 7016-5982-M001 eBiosciences</td>
</tr>
<tr>
<td>Control 1</td>
<td>Purified rat IgG</td>
<td>N/A</td>
<td>0.5mg/ml</td>
</tr>
<tr>
<td>Control 2</td>
<td>Rat IgG2a isotype control</td>
<td>Keyhole limpet haemocyanin</td>
<td>0.5mg/ml</td>
</tr>
</tbody>
</table>

Table 8: Anti-PDL1 and control antibodies used in-vivo

The antibody is drawn up into a 1ml syringe with a 29 gauge needle which is inserted in the left lower quadrant and directed towards the epigastrium at an angle of approximately 15°. When the needle is 3-4mm from the hilt the syringe plunger is retracted and if no blood is seen the dose is given through slow injection. The animal is then left to recover.
2.5 Tissue Harvesting

The animal was anaesthetised as previously described and positioned in dorsal recumbency with the limbs displaced laterally and fixed to the operating surface with adhesive tape. An 80% alcohol mixture was liberally distributed over the abdomen and a median incision made using dissecting scissors from the pelvis to the xiphisternum. The abdominal-pelvic cavity was held open using retractors and the bowel positioned laterally to facilitate harvesting (see Figure 18).

The blood was drained using a 21 gauge needle attached to a 3ml syringe via direct puncture at the junction of the inferior vena cava and portal vein. The whole blood is left to rest for 1 hour in an eppendorf tube prior to centrifugation at 5,000g for 5 minutes (Microfuge® 22R Centrifuge, Beckman Coulter, Brea, CA) to separate the serum for analysis. The spleen is located in the left upper quadrant of the abdomen and is easily dissected from its vascular pedicle and the pancreas. The liver is removed en-bloc by cutting it free of its pedicle and surrounding ligaments.
The nodes of importance in draining the liver were then harvested. In mice, the lymph nodes preferentially draining the liver have been identified as the portal, coeliac and first mesenteric chain [590, 591]. Evans Blue dye injected into the liver principally migrates to these nodes, whereas dendritic cell migration from the liver and subsequent activation of T cells appears to concentrate into the portal and coeliac nodes [590]. Anatomically, the portal and coeliac nodes are located in close proximity...
to the junction of the inferior vena cava and portal veins, behind the stomach. The first mesenteric chain is the most proximal group of nodes within the mesentery of the jejunum.

For some animals the allograft skin and allograft draining lymph nodes (brachial, inguinal and cervical) were also collected. The brachial and inguinal lymph nodes are best collected with the animal positioned on its side and an area of skin dissected from the junction of the limb and thorax. These nodes lie quite superficially and can often be seen as a darkened area in adipose tissue which can be dissected out under microscope-assisted vision. The cervical nodes are easily located in the anterolateral neck with the animal in the dorsal position. Depending on the tissue processing strategy, samples were either placed in cell culture medium to be processed for flow cytometric analysis, cut into small ~2x2mm segments and snap frozen in liquid nitrogen before storing in cryovials for RNA extraction, or set in Tissue-Tek® OCT™ Compound (Sakura Finetek USA, Torrance, CA) and frozen in liquid nitrogen for subsequent sectioning and staining. Cryovials were stored in liquid nitrogen and serum and OCT blocks in a -80°C freezer.

2.6 CUTTING FROZEN SECTIONS

Frozen sections of the liver and spleen were cut on a Cryotome E Cryostat (Thermo Scientific, Waltham, MA) set at -15°C. Sections were cut at 6μm width and placed onto labelled Menzel-Gläser Superfrost® Plus slides (Lomb Scientific, NSW, Australia) then left to air-dry at room temperature for 1 hour. The slides were then fixed in 100%
acetone for 8 minutes in Coplin jars and left to air-dry at room temperature for 10 minutes. Slides were packed in foil then labelled and stored in a -80°C freezer prior to staining.

2.7 TISSUE STAINING AND SLIDE PREPARATION

Haematoxylin and eosin staining was performed on the livers of harvested mice to examine cellular architecture and inflammatory infiltrates. This was outsourced to the Histopathology Laboratory at the Blackburn Building, University of Sydney

Harvested samples were stained for K<sup>+</sup>, FoxP3, CD4, CD8a, F4/80, B220 and PD-L1 using either fluorescein isothiocyanate (FITC) conjugated or biotinylated antibodies to assess the efficiency of inoculation, inflammatory response and/or upregulation of ligands.

2.7.1 STOCK SOLUTIONS FOR IMMUNOHISTOCHEMISTRY STAINING

Stock solutions for IHC staining were prepared as follows:

- **TBST** x 10 (Tris-Buffered Saline, 0.5% Tween-20) stock was made from 13.9g Tris Base (Sigma), 60.6g Tris-HCl (Sigma), 60.6g NaCl (UNIVAR) and 5ml 0.5% Tween-20 (AMRESCO, Solon, OH) made up to 1L with TDW and adjusted to pH 7.4. Stock was stored at 4°C and diluted 1:10 in TDW prior to use.

- **IP Diluent** stock (50mls) was made from 47.25ml TBST, 2.5ml of heat-inactivated swine serum and 0.25mls of 15% NaN<sub>3</sub> (AnalaR Normapur®, VWR
International, Radnor, PA) mixed and passed through a 0.22μm filter. Stock was stored at 4°C.

- **0.1% v/v Triton-X-100 in 0.1% w/v C₆H₇NaO₇** was made as a permeabilising agent. Stock was prepared by mixing 5mls of 2% C₆H₇NaO₇ (made by dissolving 2g of C₆H₇NaO (Sigma) in 100ml of TDW), 95mls of TDW and 100μL of Triton-X-100 (Sigma).

- **DAB (3,3′-Diaminobenzidine) was prepared by using the Liquid DAB+ Substrate Chromogen System K3468 (Dako, Carpinteria, CA) by adding 1 drop of chromogen to 1ml of substrate with 15μL of NaN₃ (AnalaR).**

### 2.7.2 INDIRECT IMMUNOHISTOCHEMICAL STAINING: FITC CONJUGATED PRIMARY ANTIBODIES

Immunohistochemical staining was performed on harvested livers for K⁺, FoxP3, CD4, CD8a, F4/80 and B220 using fluorescein isothiocyanate (FITC) conjugated antibodies. Slides were removed from the -80°C storage freezer and thawed at room temperature while still wrapped in foil (to prevent condensation) for 15 minutes. After this, the slides were taken out of the foil, placed on a staining tray and left to air-dry at room temperature for 30 minutes. All were labelled (stain, sample or control) with a graphite pencil. The staining area of each section was marked out with a wax pen (Dako, Glostrup, Denmark) and left to dry for 5 minutes. After this slides were rinsed briefly in TBST. The intra-nuclear FoxP3 stain required permeabilisation which was achieved by incubating the slides in cold 0.1% v/v Triton-X-100 in 0.1% w/v C₆H₇NaO₇ for 2 minutes in a Copplin jar, after which they were rinsed twice in TBST for 5 mins.

The other stains did not require this permeabilisation step as the antibodies bind to
cell-surface antigens, and were rinsed briefly in TBST. Slides were blocked in IP diluent containing 20% normal mouse serum (NMS) for 20 minutes by pipetting 50-100μL into the area marked out by the wax pen. The slides were then inverted to remove the blocking agent and the primary antibody was added at the specified concentration mixed with IP diluent (see Table 9). For FoxP3 staining, the primary antibody was left on for 45 minutes, for all other stains it was left on for 30 minutes. Slides were then washed twice in TBST for 5 minutes. The secondary antibody was then added and the samples incubated for 30 minutes. After this the slides were again washed twice in TBST for 5 minutes. DAB was prepared and added to the staining area followed by incubation at room temperature for 2 - 5 minutes, monitoring for a change of tissue colour. The slides were then rinsed in 3 changes of tap water. All primary antibodies were conjugated to FITC, the secondary antibody was a rabbit derived IgG against FITC conjugated to horseradish peroxidase (HRP). The peroxidase enzyme catalyses the conversion of chromogenic DAB substrate into a coloured product, revealing where the primary antibody has bound.

Counterstaining was performed while the slides were still wet. One method used was to immerse the slides in Mayer’s haematoxylin at room temperature for 2 minutes and then rinse in 3 changes of tap water. Alternatively, the slides were immersed in haematoxylin for 10 seconds then rinsed in water, followed by 3 quick dips in acid alcohol to optimise cytoplasmic differentiation. Following this the slides were rinsed in water and then immersed in Scott’s blueing solution for 30 seconds.

Dehydration was performed in graded alcohols: 70% ETOH for 1 minute, 95% ETOH for 1 minute, 95% ETOH for 1 minute, 100% ETOH for 1 minute, 100% ETOH for 1 minute
then histolene twice for 2 minutes. Coverslips were mounted with DPX (BDH Prolabo, VWR International, Poole, England) and the slides left to dry for 24 hours before examination (see Figure 19).

Figure 19: Performing IHC staining and coverslipping
<table>
<thead>
<tr>
<th>Stain</th>
<th>Antibodies</th>
<th>Target</th>
<th>Dilution</th>
<th>Clone</th>
</tr>
</thead>
<tbody>
<tr>
<td>K&lt;sup&gt;b&lt;/sup&gt;</td>
<td>FITC mu anti-muH-2K&lt;sup&gt;b&lt;/sup&gt;</td>
<td>MHC class I K&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1:100 (at 0.5mg/ml)</td>
<td>Clone: AF6-88.5 Cat: 553569 Lot: 59410 BD Biosciences</td>
</tr>
<tr>
<td>Isotype</td>
<td>FITC muIgG2aκ isotype</td>
<td>N/A</td>
<td>1:100 (at 0.5mg/ml)</td>
<td>Cat: 553456 Lot: 78136 BD Biosciences</td>
</tr>
<tr>
<td>FoxP3</td>
<td>FITC conjugated ratlgG2a</td>
<td>N/A</td>
<td>1:50 (at 0.5mg/ml)</td>
<td>Clone: eBR2a Cat: 11-4321-81 Lot: E028400 eBiosciences</td>
</tr>
<tr>
<td>CD4</td>
<td>FITC conjugated rat lgG2b</td>
<td>N/A</td>
<td>1:100 (at 0.5mg/ml)</td>
<td>Clone: eB149/10H5 Cat: 11-4031-81 Lot: E031429 BD Biosciences</td>
</tr>
<tr>
<td>CD8a</td>
<td>FITC conjugated rat lgG2a</td>
<td>N/A</td>
<td>1:50 (at 0.5mg/ml)</td>
<td>Clone: 53-6-7 Cat: 553031 Lot: 81370 BD Biosciences</td>
</tr>
<tr>
<td>B220</td>
<td>FITC conjugated rat lgG2a</td>
<td>N/A</td>
<td>1:50 (at 0.5mg/ml)</td>
<td>Clone: RA3-6B2 Cat: 553087 Lot: 50450 BD Biosciences</td>
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<tr>
<td>F4/80</td>
<td>FITC conjugated anti-muF4/80</td>
<td>Macrophages, Kupffer cells</td>
<td>1:400 (at 0.5mg/ml)</td>
<td>Clone: BM8 Cat: 11-4801-82 Lot: E00611-413 eBiosciences</td>
</tr>
<tr>
<td>Isotype</td>
<td>FITC conjugated rat lgG2a</td>
<td>N/A</td>
<td>1:50 (at 0.5mg/ml)</td>
<td>Clone: eBR2a Cat: 11-4321-81 Lot: E028400 eBiosciences</td>
</tr>
<tr>
<td>For all Secondary</td>
<td>rab anti-FITC HRP IgG</td>
<td>FITC</td>
<td>1:200 (1mg/ml)</td>
<td>Cat: 18-783-77622 Lot: 200910 Genway</td>
</tr>
</tbody>
</table>

Table 9: Antibodies used for immunohistochemistry
2.7.3 INDIRECT IMMUNOHISTOCHEMICAL STAINING: BIOTINYLATED PRIMARY ANTIBODY

Staining for PD-L1 was performed using a biotinylated antibody. Protocol optimisation was required and changes made to improve the protocol included:

- Changing the buffer solution from phosphate buffered saline (PBS) to TBST
- Removing buffer rinsing steps after blocking
- Changing the brand of antibody and isotype control used
- Trialling different antibody concentrations
- Removing a H₂O₂ blocking step which caused significant tissue architecture disruption at a range of concentrations

In the optimised protocol, slides were removed from storage in the -80°C freezer and left on the staining rack whilst still wrapped in foil for 15 minutes. The slides were then removed and ordered as desired on the rack and labelled then left to dry for 30 minutes. Sections were demarcated with a wax pen (Dako, Glostrup, Denmark) and once the wax had dried, the slides were washed in TBST 3 times for 3 minutes. Excess solution was removed by gently tapping the base of the slide onto paper towel. Biotin blocking was performed using the Biotin Blocking System X0590 (Dako, Carpenteria, CA). Sections were incubated with avidin solution (Dako) for 30 minutes to block endogenous biotin. After removing excess avidin solution, the slides were incubated with biotin solution to block residual avidin for 10 minutes (Dako). Excess biotin solution was removed by inverting the slides and tapping them gently onto paper towel. The slides were blocked with TBST + 20% NHS (normal horse serum) for 20 minutes. The excess block was removed by gently tapping the base of the slides onto some paper towel before adding the antibody (either anti-PDL1 or isotype control see
Table 10) diluted to the appropriate concentration in TBST with 1% NHS. Slides were left to incubate for 1 hour. During the incubation phase, the detection solution was prepared using the Vectasatin ABC Kit PK-4000 (Vector Laboratories, Burlingame, CA) by mixing 1:100 of reagent A and 1:100 of reagent B into TBST with 1% NHS and leaving the solution to rest for 30 minutes. After the antibody incubation was complete the slides were rinsed in TBST 3 times for 3 minutes and the detection solution added for 30 minutes. The DAB chromogen solution was prepared whilst the detection reagent was incubating. The slides were then rinsed in TBST 3 times for 3 minutes before applying the chromogen solution to the sections for 2 - 5 minutes (until sections turned brown), after which the slides were rinsed in water. Counterstaining and cover-slippering was performed as described above (2.7.1 Indirect immunohistochemical staining: FITC conjugated primary antibodies).

<table>
<thead>
<tr>
<th>Stain</th>
<th>Antibody</th>
<th>Target</th>
<th>Dilution</th>
<th>Clone</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD-L1 control</td>
<td>Anti-mu CD274 biotin</td>
<td>Programmed Cell Death Ligand 1</td>
<td>1:25,50,100 (at 0.5mg/ml)</td>
<td>Clone: MIH5 Cat: 13-5982-85 Lot: E03115-1630 eBiosciences</td>
</tr>
<tr>
<td>PD-L1 control</td>
<td>Rat IgG2α biotin</td>
<td>Keyhole limpet haemocyanin</td>
<td>1:25,50,100 (at 0.5mg/ml)</td>
<td>Clone: eB2a Ref: 13-4321-81 Lot: E02841-1630 eBiosciences</td>
</tr>
<tr>
<td>Antibody</td>
<td>Anti-mu CD274 biotin</td>
<td>Programmed Cell Death Ligand 1</td>
<td>1:50 (at 0.5mg/ml)</td>
<td>Clone: 10F.9G2 Cat: 124306 Lot: B148620 BioLegend</td>
</tr>
<tr>
<td>Isotype control</td>
<td>Rat IgG2β biotin</td>
<td>N/A</td>
<td>1:50 (at 0.5mg/ml)</td>
<td>Clone: RTK4530 Cat: 400604 Lot: B168726 BioLegend</td>
</tr>
</tbody>
</table>

Table 10: Biotinylated antibodies
2.8 Hepatocyte Isolation for Flow Cytometry

Hepatocytes were isolated by liver perfusion as described by Bowen et al. [592] (modified from Seglen [593]) from B10.BR mice at days 2, 7 and 14 post-inoculation with rAAVkb or rAAVD227K 5 x 10^{11} vgc. The animal was anaesthetised as previously described, a 22 gauge cannula introduced into the inferior vena cava, and the portal vein transected. Four 50ml Falcon tubes containing 25mls of 1) Hank’s BSS (Gibco Life Technologies, Carlsbad, CA), 2) Hank’s BSS + 0.5mM EDTA (Sigma), 3) Hank’s BSS and 4) Hank’s BSS with 5mM CaCl_2 (UNIVAR) and 0.05% collagenase IV (Sigma-C5138) were warmed in a TW8 water bath (Julabo, Allentown, PA) at 37°C. A line attached to a MasterFlex L/S peristaltic pump (Cole-Parmer, Vernon Hills, IL) set to a flow rate of 5mls/minute was primed with Hank’s buffered salt solution from tube 1). The line was then connected to the cannula (being careful not to introduce any air into the system) and the liver serially perfused with the solutions from tubes 1 through 4. The gallbladder was excised from the liver and the liver subsequently harvested and placed in a petrie dish containing RPMI 1640 (Gibco)/10% FCS (fetal calf serum) (Sigma). The hepatocytes were teased from the liver into the medium with the back of a scalpel blade and subsequently precipitated by two centrifugations in RPMI 1640/10% FCS at 50g for 3 minutes at 4°C. To enrich for live cells, a Percoll gradient centrifugation was performed by resuspending the isolated hepatocytes in 15mls of PBS with 9mls of isotonic Percoll (GE Healthcare, Little Chalfont, UK). This was centrifuged at 500g for 15 minutes at 20°C with the brake off in a Microfuge 22R® Centrifuge (Beckman Coulter) and the aggregated material and supernatant discarded. The pellet of live cells was washed twice in RPMI/10% FCS.
2.9 Flow Cytometry - FACS

Flow cytometry was performed on isolated hepatocytes to assess vector and PD-L1 expression. Cells were counted and viability assessed by exclusion of trypan blue (Life Technologies). The cell pellet was resuspended in cold staining buffer (cold PBS containing 2% fetal calf serum and 0.1% NaN₃) at a concentration of 2 x 10⁷ cells/ml. Primary antibodies (see Table 11) were diluted to working concentrations in staining buffer in FACS tubes, then 1 x 10⁶ live cells delivered to each tube. The tubes were gently vortexed to facilitate mixing and then incubated for 30 minutes at 4°C in the dark. The cells were subsequently washed twice with 2mls of staining buffer. The cell pellet was resuspended in 100μL of secondary antibody diluted 1:200 and the tubes gently vortexed prior to incubation for 30 minutes at 4°C in the dark. The cells were subsequently washed twice with 2mls of cold staining buffer before resuspending the final pellet in 400-500μL of PBS. Samples were acquired on an LSR II flow cytometer using FACSDiva software (BD Biosciences, Mountain View, CA) and analysis performed using FlowJo Version 10.0.7 (Tree Star, Ashland, OR).
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Target</th>
<th>Dilution</th>
<th>Clone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti H2-αK(^b) biotinylated (Primary)</td>
<td>MHC class I K(^b) (α1, α2, α3 domains)</td>
<td>1:100 (at 0.5mg/ml)</td>
<td>Clone: AF6-88.5 Cat: 553569 Lot: 59410 &lt;br&gt;BD Biosciences</td>
</tr>
<tr>
<td>Anti H2-αK(^b) biotinylated (Primary)</td>
<td>MHC class I K(^b) (α1, α2 domains)</td>
<td>1:100 (at 1mg/ml)</td>
<td>Clone: B8.24.2 WEHI</td>
</tr>
<tr>
<td>Anti H2-αK(^b) biotinylated (Primary)</td>
<td>MHC class I K(^b) (α1, α2, α3 domains)</td>
<td>1:100 (at 0.5mg/ml)</td>
<td>Clone: K9-178 WEHI</td>
</tr>
<tr>
<td>Anti-mouse CD274 biotinylated (Primary)</td>
<td>Programmed Cell Death 1 Ligand 1</td>
<td>01:100 (at 0.5mg/ml)</td>
<td>Clone: MIH5 Cat: 7016-5982-M001 Lot: E17964-101 ebiosciences</td>
</tr>
<tr>
<td>PE Streptavidin (Secondary)</td>
<td>Biotin</td>
<td>1:200 0.5mg/ml</td>
<td>Cat: 554061 Lot: 3017962 Material: 554061 BD Biosciences</td>
</tr>
</tbody>
</table>

Table 11: Primary antibodies used in FACS

2.10 **Statistical Analysis**

Statistical analysis was performed using Prism 5 (GraphPad, La Jolla, CA) software. Data graphed in columns represents the mean of the data set, with the error bar displaying the standard error of the mean. Groups of data were compared by performing a one way analysis of variance followed by post hoc Tukey’s multiple comparison tests with a significance level of p < 0.05. Survival curves were compared using the Log-rank (Mantel Cox) test with a significance level of p < 0.05.
3.0 RESULTS

3.1 IN-VIVO TRANSGENE EXPRESSION

Mice injected with rAAVKb at doses ranging from $5 \times 10^9$ to $2 \times 10^{12}$ vector genome copies (vgc) in our laboratory have previously been shown to exhibit liver-specific transgene expression [523]. Immunohistochemical staining for Kb in naïve B10.BR mice inoculated with $5 \times 10^{10}$ to $5 \times 10^{11}$ vgc of both rAAVKb and rAAVD227K showed strong expression of both WT-Kb and Kb-D227K on hepatocytes comparable to untreated C57BL/6 mice which constitutively express the MHC class I Kb molecule. Similarly, B10.BR animals primed by rejecting a primary 178.3 graft and inoculated with $5 \times 10^{10}$ vgc of rAAVKb 7 days after rejection also displayed successful transduction (harvested 7 and 14 days after inoculation), confirming the ability of the recombinant adenoviral vector to successful confer hepatic expression of transgenes in the face of active immunity. All animals remained healthy on serial monitoring (see Figure 20).
Flow cytometry was performed on isolated hepatocytes to quantitate expression of wild-type and mutant K\(^b\). Initial results showed an apparent reduction in expression of K\(^b\)-D227K compared with WT-K\(^b\) using the anti-K\(^b\) monoclonal antibody AF6-88.3. It was hypothesised that the reduced staining may be due to diminished antibody binding affinity as a result of the mutation introduced into the acidic loop of the MHC class I molecule in the D227K variant. A comparison between anti-K\(^b\) AF6-88.3 and K9-
178 clones (which bind to the α1 to α3 domains) as well as an anti-Kb B8.24.3 antibody (which binds only to epitopes in the α1 and α2 domains) was performed (see Figure 21). Staining for Kb expression at day 7 post injection of rAAVKb 5 x 10^{11} vgc or rAAVD227K 5 x 10^{11} vgc using the B.8.24.3 antibody revealed that expression between the two groups was comparable, confirming that both wild-type Kb and mutant Kb are strongly expressed on hepatocytes after transduction.

![AF6-88.5 clone](image1.png) ![K9-178 clone](image2.png) ![B8.24.3 clone](image3.png)

**Figure 21:** Wild-type and mutant Kb expression on isolated hepatocytes by FACS using different anti-Kb antibody clones

3.2 **Inflammatory Response to rAAV**

Staining for B cells, T cells and macrophages in the livers of mice injected with 5 x 10^{11} vgc of rAAVKb and rAAVD227K using antibodies to CD4, CD8a, B220 and F4/80 revealed no significant inflammatory infiltrates (see Figure 22).
No evidence of an inflammatory reaction noted after rAAVkb or rAAVD227K (dose 5 x 10^{11} vgc)
Serum ALTs performed 7 days after rAAVKb and rAAVKD227K inoculation did not show any statistically significant rise compared to naive B10.BR controls (see Figure 23). Taken together, these results provide evidence for that rAAVKb and rAAVD227K can be administered safely without eliciting an appreciable inflammatory response.

![Figure 23: ALTs after WT-Kb and K-D227K transduction](image)

### 3.3 Skin Graft Survival in Naïve and Transduced Animals

#### 3.3.1 Allograft Appearance in naïve and WT-Kb-transduced B10.BR Mice

A control group of naïve B10.BR mice were grafted with skin from 178.3 strain donors to establish a baseline time to rejection. Macroscopically, rejection was first noted as allograft shrinkage, dryness and elevation, with darkening of the graft. The desiccated graft subsequently detached, leaving either an open wound or underlying scar (see Figure 24). Mean survival time was 16 days.
**Figure 24: Rejection of allografts in naive recipients**

Macroscopic appearance of 178.3 allografts to naive B10.BR mice at days 0, 7, 17 and 23 showing the process of rejection and finally resolution leaving a scar.

Allografts performed on B10.BR animals treated with rAAVKb 5 x 10^{10} vgc a week prior to transplant demonstrated operational tolerance (sacrificed at 250 days post-transplant with surviving grafts). All grafts remained soft with good integration into surrounding tissues, confirming the ability of donor MHC class I expression on hepatocytes to confer long-term skin graft survival to single-MHC class I mismatched recipients. There was some disparity in the macroscopic appearance of grafts at day 250, with some retaining a completely normal appearance and others appearing slightly atrophic (see **Figure 25**).
Figure 25: Allografts to mice transduced with WT-K<sup>b</sup>

At 250 days post-transplant, allografts remained viable, with some appearing completely healthy (A) and others displaying atrophic features (B)

Haematoxylin and eosin staining revealed that allografts which appeared atrophic macroscopically show more sub-epithelial collagen and less dermal components (see Figure 26). All retained viable epithelium.
Figure 26: Allografts to mice transduced with WT-K^b

All show viable epithelium at day 250, with macroscopically healthy allografts (B) appearing to similar to
syngeneic grafts (A,C) and macroscopically atrophic allografts showing more dermal collagen (D)

3.3.2 Allograft survival curves for naïve and transduced animals

Naïve B10.BR mice receiving 178.3 grafts showed a median survival time (MST) of 16
days. In contrast, mice treated with 5 x 10^{10} vgc of rAAVKb showed operational
tolerance to 178.3 allografts placed seven days after transduction (euthanized at 250
days post-transplant) with a statistically significant survival advantage (p = 0.0002).
There was also a statistically significant prolongation in skin graft survival for animals
treated with 5 x 10^{10} vgc of rAAVD227K seven days prior compared to naïve animals
(median survival time 27 days, p = 0.0016), however tolerance was not achieved.
Increasing the dose of rAAVD227K did not result in further prolongation of graft survival: median survival time 26 days ($p = 0.0042$) (see Figure 27).

![178.3 Skin Graft Survival](image)

**Figure 27: Skin graft survivals in WT-$K^b$ and $K^b$-D227K treated mice**

(Note: graph truncated at day 100, but rAAVKb-treated B10.BR mice were still tolerant to 178.3 allografts at day 250)

One possible explanation for the slight prolongation in graft survival seen in mice expressing mutant $K^b$ is that high affinity CD8 T cells which do not require CD8 co-receptor binding and/or CD8 T cells recognising processed $K^b$ are initially activated and then functionally exhausted or deleted, leaving a population of naïve, lower affinity CD8-dependent T cells which are subsequently activated upon grafting with $K^b$-bearing skin and mediate rejection at a reduced tempo. Alternatively, high affinity alloreactive CD8 T cell clones may initially proliferate and expand to a greater extent than usual in the absence of any competition from lower affinity cells, and these expanded clones may be mediating the delayed rejection. To elucidate the mechanism
further, a cohort of animals (n = 4) were treated with $5 \times 10^{11}$ vgc of rAAVD227K and grafted 21 days later to allow more time for exhaustion of high-affinity clones to occur. This cohort is currently incomplete due to technical difficulties, with one surviving mouse showing graft rejection at day 28. A further possibility is that presentation of processed $K^b$-D227K peptides has resulted in the generation of regulatory T cells which are able to delay, but not completely prevent rejection. Experiments to determine whether regulatory T cells contribute to prolonged allograft survival in this setting will be performed in the future using DEREG mice, but are beyond the scope of this thesis.

3.3.3 Allograft appearance in $K^b$-D227K transduced B10.BR mice

Mice treated with rAAVD227K displayed a prolonged time to allograft rejection and a different process macroscopically to naïve animals. Initially, the grafts appeared thickened and elevated, with punctate areas of scabbing and sloughing of superficial epidermis. Progressively, the allograft appeared to resemble a thickened pale scab which was very adherent to the underlying tissue. The dried graft later sloughed off leaving a wound or fragile scar tissue. The more indolent pathway to rejection is clearly seen in Figure 28 below, where at 7 days post grafting the allograft is dry, dark and elevated in an untreated animal but remains soft and clearly viable in an animal treated with rAAVD227K.
Figure 28: Macroscopic appearance of rAAVD227K grafts

Distinct difference at day 7 between naïve (A) and rAAVD227K (B) treated animals, with the rAAVD227K inoculated animals showing imminent rejection at day 28 (C) with subsequent graft loss (MST 26 days), which heals to leave a small scar (D, day 104)
3.4 Induction of PD-L1 on Hepatocytes after WT-K^b and K^b-D227K Transduction

3.4.1 PD-L1 Expression on Transduced Hepatocytes

PD-L1 expression on rAAVKb transduced hepatocytes was investigated to ascertain whether it could play a role in the induction of operational tolerance. A time series at day 2, 7 and 14 post penile vein injection of rAAVKb 5 x 10^{11} vgc was performed using FACS on isolated hepatocytes (see Figure 29). Whereas strong expression of the K^b transgene was already evident at 48 hours post-inoculation, upregulation of PD-L1 on hepatocytes was not noted until day 7, and had decayed towards baseline by day 14.

Figure 29: Time-course of PD-L1 expression of rAAVKb-transduced hepatocytes
In contrast, mice treated with rAAVD227K did not reveal a rise in PD-L1 expression at any time point. Expression in the B10.BR controls or rAAVD227K-treated mice was no greater than that on unstained hepatocytes (see Figure 30).

![Figure 30: PD-L1 expression after rAAVKb versus rAAVD227K](image)

PD-L1 is upregulated strongly on K\(^b\)-transduced hepatocytes at day 7, whilst those expressing mutant K\(^b\) display no response.

Confirmatory findings were noted on IHC staining using anti-CD274 on a range of livers transduced with rAAVKb and rAAVD227K (5 x 10\(^{11}\) vgc) to investigate differences in PD-L1 ligand induction: strong expression of PD-L1 was noted on hepatocytes expressing WT-K\(^b\) but rAAVD227K-treated animals showed similar expression levels to B10.BR controls (see Figure 31). Taken together, these findings suggest that cross-talk between activated T cells and hepatocytes may be required for PD-L1 upregulation in the liver parenchyma.
PD-L1 upregulation on hepatocytes was only seen when CD8 T cells were activated by direct antigen recognition and this group of animals subsequently developed tolerance, possibly through silencing or deleting reactive CD8 T cells via PD-1:PD-L1 interactions. When direct antigen recognition by CD8 T cells was abrogated by site-directed mutagensis of the K\(^b\) molecule, PD-L1 was not upregulated.

3.4.2 PD-L1 UPREGULATION IS DEPENDENT ON EXPRESSION OF A TRANSGENE WHICH CAN BE RECOGNISED BY CD8 T CELLS

To further test the hypothesis that upregulation of PD-L1 is dependent on recognition of the transgene product by CD8 T cells and not due to the vector itself, FACS was performed on isolated hepatocytes from C57BL/6 mice inoculated one week prior with rAAV Kb. As C57BL/6 mice naturally express the K\(^b\) molecule, any immune response will be to an antigenic stimulus from the vector itself and not the transgene product. No rise in PD-L1 expression was noted (see Figure 32). Conversely, inoculation of C57BL/6 mice with a viral vector expressing the WT-K\(^d\) allele does elicit PD-L1 upregulation, confirming that the response is due to expression of the

Figure 31: Anti-PDL1 stain (liver tissue x 20)
allogeneic transgene product. Using the same platform, WT-K<sup>d</sup> transduction of CD57BL/6 Rag mice does not produce upregulation of PD-L1 on hepatocytes, confirming that the response is mediated via lymphocytes (data not shown).

![Figure 32: PD-L1 expression after rAAVKb transduction in C57BL/6 mice](image)

To determine whether PD-L1 upregulation on hepatocytes was required for inactivation of alloreactive CD8 T cells and/or subsequent acceptance of K<sup>b</sup>-bearing skin grafts, we proceeded to a series of in-vivo experiments using blocking antibodies against PD-L1.
3.5 **The Effect of PD-L1 Blockade on Kb-Transduced Livers and Allograft Survival**

Given the possibility that PD-L1 blockade may trigger hepatitis due to a failure to silence Kb-reactive T cell clones, a series of animals were inoculated with either rAAVKb and anti-PDL1, anti-PDL1 alone or rAAVKb and control antibody to assess the safety of PD-L1 blockade prior to performing skin graft procedures (see Table 12).

<table>
<thead>
<tr>
<th>Mice</th>
<th>Inoculations</th>
<th>Harvested</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B10.BR (n = 3)</strong></td>
<td>Day 0: rAAVKb 5 x 10^{10} and 500mcg anti-PDL1</td>
<td>Day 7</td>
</tr>
<tr>
<td></td>
<td>Day 3: 250mcg anti-PDL1</td>
<td></td>
</tr>
<tr>
<td><strong>B10.BR (n = 3)</strong></td>
<td>Day 0: rAAVKb 5 x 10^{10} and 500mcg control 1</td>
<td>Day 7</td>
</tr>
<tr>
<td></td>
<td>Day 3: 250mcg control 1</td>
<td></td>
</tr>
<tr>
<td><strong>B10.BR (n = 3)</strong></td>
<td>Day 0: Kb 5 x 10^{10} and 500 mcg control 2</td>
<td>Day 7</td>
</tr>
<tr>
<td></td>
<td>Day 3: 250mcg control 2</td>
<td></td>
</tr>
<tr>
<td><strong>B10.BR (n = 3)</strong></td>
<td>Day 0: 500mcg anti-PDL1</td>
<td>Day 7</td>
</tr>
<tr>
<td></td>
<td>Day 3: 250mcg anti-PDL1</td>
<td></td>
</tr>
</tbody>
</table>

**Table 12:** Inoculations performed on B10 mice to assess safety of PD-L1 blockade
3.5.1 Immediate PD-L1 blockade in mice transduced with rAAVKb results in moderate hepatitis

Determination of serum alanine aminotransferase (ALT) was performed on control B10.BR mice, those treated with rAAVKb alone and those treated with rAAVKb and either control antibody or anti-PDL1 (see Figure 33). Those receiving rAAVKb or anti-PDL1 alone showed no statistically significant increase in ALT levels. Mice treated with rAAVKb and either control antibody showed a mild rise in ALT which was statistically significant (p ≤ 0.05). Due to initial findings of increased inflammatory infiltrates and raised ALTs in mice treated with control 1 (purified rat IgG), a more specific functional grade isotype control 2 (rat IgG2α) directed against the irrelevant antigen keyhole limpet haemocyanin was also trialled in an attempt to remove the possibility of endotoxin contamination or cross-reactivity. This had no appreciable effect and results from each group receiving control antibody were similar, allowing the groups to be merged for analysis. Animals inoculated with rAAVKb and anti-PDL1 showed a marked rise in ALT (p ≤ 0.001) with a mean value of 721 ± 73 U/L. All animals remained well and active on serial monitoring.

![Serum ALT](image)

Figure 33: Serum ALT levels after PD-L1 blockade
3.5.2 **MACROSCOPIC APPEARANCE OF LIVERS**

Macroscopically, hepatic lesions were noted in mice treated with either rAAVKb and anti-PDL1 or rAAVKb and isotype control (see **Figure 34**).

![Figure 34: Macroscopic appearance of livers under PD-L1 blockade](image)

Multiple lesions were noted in mice injected with rAAVKb and either anti-PDL1 or control antibody, while mice treated with rAAVKb or anti-PDL1 alone had macroscopically normal livers.

In the rAAVKb with anti-PDL1 group, the lesions were comprised of punctate areas with central haemorrhage, while those injected with rAAVKb and control antibody showed a few scattered lesions with a pale appearance. Mice treated with anti-PDL1 alone or rAAVKb alone showed no macroscopic abnormalities.
3.5.3 Peri-portal infiltrates are present in mice treated with rAAVkb and either anti-PDL1 or control antibody

Haematoxylin and eosin staining revealed an increase in cellular infiltrate in mice inoculated with rAAVkb and anti-PDL1, mostly around the portal tracts (see Figure 35). Mice treated with rAAVkb and either control antibody also manifested an inflammatory reaction characterised by peri-portal and intra-lobular nodular inflammation corresponding to the lesions seen macroscopically. This was not as prominent as in the group of mice treated with both rAAVkb and anti-PDL1. Those injected with either rAAVkb alone or anti-PDL1 alone had normal tissue architecture without significant infiltrates.

![Kb + anti-PDL1](image1)
![Anti-PDL1](image2)
![Kb + control 1](image3)
![Kb + control 2](image4)
3.5.4 CHARACTERISATION AND QUANTIFICATION OF THE CELLULAR INFILTRATE IN MICE RECEIVING RAAVKb AND CONCOMITANT PD-L1 BLOCKADE

Immunohistochemical staining in the parenchyma (p) and portal tracts (pt) for inflammatory infiltrates revealed an increase in the number of inflammatory cells in mice treated with rAAVKb and anti-PDL1 or rAAVKb and control antibody, with the rAAVKb/anti-PDL1 group showing a much stronger response. Most striking was the marked upregulation of F4/80 positive cells seen around the portal tracts. Mice treated with anti-PDL1 alone did not demonstrate an inflammatory reaction, confirming that PD-L1 blockade per se does not elicit autoimmune hepatitis. Cell counting was performed in both the parenchyma and portal tracts for CD4, CD8 and B220 stains (see Figure 36). Note: cell counting was not performed for F4/80 where
the F4/80\(^+\) cells around the portal tracts and in the inflammatory nodules were too numerous to count.

<table>
<thead>
<tr>
<th></th>
<th>Parenchyma</th>
<th>Portal tracts</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CD4</strong></td>
<td></td>
<td>***</td>
</tr>
<tr>
<td><strong>CD8</strong></td>
<td></td>
<td>***</td>
</tr>
<tr>
<td><strong>B220</strong></td>
<td></td>
<td>**</td>
</tr>
</tbody>
</table>

**Figure 36: Cell counts of liver tissue under of PD-L1 blockade**

Cellular infiltrates noted in \(k^b\)-transduced animals receiving both anti-PDL1 and isotype control antibody.

Those under PD-L1 blockade demonstrate a stronger response most markedly around the portal tracts

Staining for CD4, CD8, B220 and F4/80 is shown in Figures 37 to 40.
Figure 37: PD-L1 blockade - F4/80 stain (liver x 20)
Figure 38: PD-L1 blockade - CD4 stain (liver x 20)
Figure 39: PD-L1 blockade – CD8 stain (liver x 20)
Figure 40: PD-L1 blockade - B220 stain (liver x 20)
The scattered intra-lobular lesions seen in mice treated with rAAVKb and control antibody stained strongly for F4/80, presumably reflecting aggregations of macrophages (see Figure 41). There was a mild infiltrate of CD4+ cells along with scattered B220+ cells, reflecting CD4 T cells and B cells (note B220 also stains for apoptotic CD8 cells).

Figure 41: Inflammatory hepatic nodule after rAAVKb and control antibody

Scattered CD4+ (x 20) cells with few CD8+ (x 20) or B220+ (x 40) cells, but marked F4/80 (x 40) staining
3.5.6 In vivo $K^b$ expression is maintained after immediate PD-L1 blockade

Immunohistochemical staining was performed for $K^b$ to ascertain the effect of anti-PDL1 administration on rAAV$K^b$ transduction (see Figure 42).

Figure 42: $K^b$ expression under PD-L1 blockade (liver tissue x 20)
Despite biochemical evidence of hepatitis and the presence of an inflammatory infiltrate, mice injected with rAAVKb $5 \times 10^{10}$ vgc and control antibody or anti-PDL1 retained high levels of Kb expression comparble to naïve C57BL/6 mice which consitutively express Kb.

3.5.7 DELAYING PD-L1 BLOCKADE ON KB-TRANSUCED LIVERS MITIGATES THE INFLAMMATORY RESPONSE

Given the inflammatory reaction seen with immediate PD-L1 blockade in the context of rAAVKb transduction, a series of animals were given delayed blockade when PD-L1 expression on hepatocytes had decayed (> 14 days). Animals were inoculated with rAAVKb ($5 \times 10^{10}$ vgc) and then administered anti-PDL1 or functional grade rat IgG2α isotype control antibody 21 and 24 days later (see Table 14).

<table>
<thead>
<tr>
<th>Mice</th>
<th>Inoculations</th>
<th>Harvest</th>
</tr>
</thead>
<tbody>
<tr>
<td>B10 (n = 3)</td>
<td>Day 0: rAAVKb $5 \times 10^{10}$ vgc</td>
<td>Day 28 (n = 3)</td>
</tr>
<tr>
<td></td>
<td>Days 21: 500μL anti-PDL1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Days 24: 250μL anti-PDL1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Day 0: rAAVKb $5 \times 10^{10}$ vgc</td>
<td></td>
</tr>
<tr>
<td>B10 (n = 3)</td>
<td>Day 21: 500μL isotype control 2</td>
<td>Day 28 (n = 3)</td>
</tr>
<tr>
<td></td>
<td>Days 24: 250μL control 2</td>
<td></td>
</tr>
</tbody>
</table>

Table 13: Timing of inoculations for delayed PD-L1 blockade
The significant rise in ALTs seen in transduced animals undergoing immediate blockade with anti-PDL1 was not seen in the delayed blockade group (see Figure 43). By day 28, ALTs in the delayed blockade group were comparable to those seen in animals treated with anti-PDL1 and control antibody which showed a sustained elevation across both time points. This suggests that while there is a non-specific reaction to giving an intact antibody in both groups. The marked inflammatory response in the rAAV Kb treated mice under immediate blockade group is a direct consequence of the present of PD-L1 on hepatocytes.

![Timecourse of Serum ALTs](image)

**Figure 43:** Time-course of ALTs in Kβ-transduced animals under PD-L1 blockade

### 3.5.8 Immediate PD-L1 Blockade Does Not Trigger Acute Rejection in rAAV Kb-Transduced Skin Graft Recipients

A series of 178.3 skin graft procedures was performed to ascertain if PD-L1 blockade could break the tolerance demonstrated in B10 recipient mice treated with 5 x 10^{10}
vgc of rAAVkb. Anti-PDL1 antibody was given at time of rAAVkb administration and for up to 2 weeks post transplant (see **Table 14**).

<table>
<thead>
<tr>
<th>Mice</th>
<th>Inoculations</th>
<th>Skin Grafts (Day 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B10 (n = 6)</td>
<td>Day 0: rAAVkb 5 x 10⁴⁰ and 500μL anti-PDL1</td>
<td>178.3 donor (n = 5)</td>
</tr>
<tr>
<td></td>
<td>Days 3, 7, 10, 14, 17, 21: 250μL anti-PDL1</td>
<td></td>
</tr>
<tr>
<td>B10 (n = 6)</td>
<td>Day 0: rAAVkb 5 x 10⁴⁰ and 500μL rat control 2</td>
<td>178.3 donor (n = 2)</td>
</tr>
<tr>
<td></td>
<td>Days 3, 7, 10, 14, 17, 21: 250μL control 2</td>
<td></td>
</tr>
</tbody>
</table>

**Table 14: Timing of inoculation and grafting for immediate PD-L1 blockade**

(Note: a combination of post-operative deaths and technical failures reduced the number of animals subsequently grafted)

Kᵇ-transduced mice demonstrated acceptance of 178.3 skin grafts under PD-L1 blockade (see **Figure 44**). At the time of submission, oldest allograft ages were 91 (n = 2) and 56 (n = 2) days with no evidence of rejection. Mice treated with rAAVkb and control antibody showed acceptance of the allograft as expected (n = 2).
3.6 **K\textsuperscript{b} Transduction in Primed Animals: Evidence for Treg Involvement in Tolerance Induction**

3.6.1 **In Vivo Expression of K\textsuperscript{b} in Primed Animals**

Previous studies from our laboratory had neither supported nor excluded a role for regulatory T cells in the induction of tolerance following rAAVKb transduction of naïve B10.BR recipients. Regulatory T cells preferentially migrate to sites of inflammation and we hypothesised that they may play a role in limiting damage to transduced hepatocytes when rAAVKb was administered to mice previously primed by rejection of a K\textsuperscript{b}-bearing skin graft. A series of naïve B10.BR animals were primed to the K\textsuperscript{b} antigen by 178.3 skin allografts, and then inoculated one week later with $5 \times 10^{10}$ vgc of rAAVKb. Groups were harvested at 7 and 14 days post-inoculation (each group also contained a primed control animal which was harvested at the same time but did not receive a dose of vector). IHC staining for K\textsuperscript{b} revealed strong cell-surface expression
comparable to that in untreated C57BL/6 mice which constitutively express MHC class I K\(^b\), whilst naive B10.BR (k-haplotype) mice showed no expression of K\(^b\) (see Figure 20).

3.6.2 IMMUNE RESPONSE TO rAAVKb IN PRIMED ANIMALS

Primed control animals showed no statistically significant rise in serum ALTs compared to naïve controls at either time point. Primed animals which subsequently received rAAVKb exhibited raised ALTs of 106 ± 24 U/L which was statistically significant (p < 0.05) (see Figure 45), yet levels remained below that consistent with overt hepatitis. All animals remained well up until time of harvest.

![Figure 45: Serum ALTs in primed mice](chart)

We examined the livers of primed mice after rAAVKb by immunohistochemical staining for B cells, T cells and macrophages. Cell counting was performed to quantify the extent of inflammatory cell infiltrate (see Figure 46). Primed animals harvested 7
days after inoculation of rAAVKb revealed a slight elevation in inflammatory infiltrate as seen by increased numbers of CD4, CD8, B220 and F4/80 positive cells. By day 14 post-inoculation the relative infiltrates of CD4, F4/80 and B220 positive cells were similar to un-injected primed control animals, though levels of CD8 remained mildly elevated. Representative sections for both time points reveal no evidence of hepatitis (see Figures 47, 48).

![Graphs of CD8, CD4, F4/80, and B220 cell counts](image)

**Figure 46: Cell counts in rAAVKb treated animals**

Each group is comprised of two mice injected 7 days after rejection of their skin transplant and then harvested 7 or 14 days after injection (Primed_inj) and one un-injected mouse received a transplant but was not inoculated with rAAVKb (Primed).
Figure 47: CD4+ and CD8+ cells in primed animals given rAAVKb (liver x 10)

rAAVKb administered 7 days after graft rejection, animals harvested at 7 and 14 days after rAAVKb administration
Figure 48: B220+ and F4/80+ cells in primed animals given rAAVKb (liver x 10)

rAAVKb administered 7 days after graft rejection, animals harvested at 7 and 14 days after rAAVKb administration
3.6.3 FoxP3 expression after WT-Kb transduction in primed animals

One pathway which may be involved in tolerance induction is the presentation of K<sup>b</sup>-derived peptides to CD4 T cells by recipient APCs resulting in the induction of regulatory T cells. Other studies have demonstrated increased pools of Tregs after hepatocyte transduction which mediate antigen-specific CD8 T cell suppression [531, 533, 534]. Staining for FoxP3 expression in primed animals was performed to look for evidence of Treg induction. Primed animals which subsequently received rAAVKb demonstrated an increase in FoxP3+ cells mainly localised to the portal tracts, whereas expression of FoxP3 in the livers of control primed mice was negligible (see Figure 49).

Figure 49: FoxP3 expression in primed animals after rAAVKb (liver x 20)

A: B6 spleen (positive control) B,C: Vector given 7 days after rejection and liver harvested 7 (B) or 14 (C) days after injection D: Primed control animal
Cell counts confirmed increased numbers of peri-portal FoxP3+ T cells in primed animals treated with rAAV Kb, most pronounced at day 7 concurrent with the peak of CD8 T cell infiltration (see Figure 50). These findings suggest that FoxP3+ Tregs may migrate to the liver in response to inflammation produced by CD8 effector-memory T cells and act to restrain hepatocyte damage as well as contribute to the development of operational tolerance in this setting.

![Graphs showing FoxP3 random fields and portal tracts](image)

**Figure 50: Cell counts after rAAV Kb in primed animals**

Groups are comprised of two mice injected 7 days after rejection of their skin transplant and then harvested 7 or 14 days after injection (Primed) and one un-injected mouse per time point which has received a transplant but was not inoculated with rAAV Kb (Primed).
4.0 DISCUSSION

Whilst infections by pathogenic microbes stimulate an immune response, self-antigens are constitutively expressed in healthy individuals and seen on resting APCs in the absence of a reactive environment. Self-reactive clones are therefore biased towards tolerance, undergoing central deletion or clonal exhaustion, anergy, deletion or suppression via regulatory T cells in the periphery. By contrast, lymphocytes targeting pathogenic peptides are biased towards proliferation and maturation. The ability to tolerise to neoantigens must therefore be a process of active immunosuppression.

There is a central role for active immunosuppression in the liver tolerance effect, as witnessed by the persistence of hepatotropic viral infections in the immunocompetent host. Subjects reveal elevated pools of regulatory T cells which mediate suppression of virus-specific CD8 cells and produce TGF-β and IL-10 which are integral to the immunosuppressive cytokine milieu [594-596]. In such cases, PD-L1 upregulation on hepatocytes and engagement of PD-1 on T cells leading to antigen-specific T cell suppression has been demonstrated [375-380]. Liver-targeted rAAV therapy exploits this unique ability of the liver, repeatedly demonstrating tolerance to a range of transgene products. Such cases are also associated with an increase in antigen-specific regulatory T cell populations which govern CD8 responses [534]. Deletion or adoptive transfer of this transgene-specific Treg population has been shown to break or confer tolerance, respectively [540]. While the regulatory T cell population is critical to maintaining tolerance, the processes by which this population is induced have not been elucidated. In the liver, there are an abundance of cells capable of priming T cell
responses including dendritic cells, Kupffer cells, hepatic stellate cells (Ito cells), liver sinusoidal epithelial cells, hepatocytes and macrophages [430, 432, 597]. The complexity of antigen presentation within the liver makes dissecting the roles of individual cell types in tolerance induction a difficult task.

4.1 $K^b$-TRANSDUCTION OF RECIPIENT HEPATOCYTES CONFERS TOLERANCE TO $K^b$-BEARING SKIN GRAFTS

Administration of the optimised rAAVKb vector ($5 \times 10^{10}$vgc) to naïve B10.BR mice yielded high-level, liver-specific expression of the transgene product on hepatocytes. $K^b$ expression was similar that in the livers of wild-type C57BL/6 mice. FACS analysis of isolated hepatocytes post rAAVKb injection demonstrated widespread expression as early as day 2 post-inoculation. Livers harvested from these animals showed no evidence of hepatitis on histological analysis. The safety of the rAAVKb vector has also been previously confirmed by our group at doses ranging from $5 \times 10^9$ to $2 \times 10^{12}$ [523]. Furthermore, animals primed by a receiving 178.3 allograft and subsequently injected with rAAVKb at day 7 post graft rejection did not show liver damage at days 7 and 14 post-inoculation, confirming that rAAVKb is safe even in the primed host.

Skin allografts from 178.3 to naïve B10.BR mice were rejected with a median survival time of 16 days (range 14 - 20 days). In this case, donor APCs (chiefly Langerhans cells) migrate from the graft to recipient secondary lymphoid tissues and activate $K^b$-specific alloreactive lymphocyte populations which proliferate and mediate destruction of the graft (see Figure 51). In contrast, skin grafts from 178.3 to B10.BR
strain animals inoculated 7 days prior with rAAVKb (5 x 10^{10} vgc) confirm our previous finding that strong expression of K^b on recipient hepatocytes confers operational tolerance across a single class I MHC allele mismatch, with grafts still viable at 250 days post-transplant.

The development of tolerance may be due to deletion, exhaustion or anergy of reactive clones, suppression via iTregs or some combination of these mechanisms. Strong, widespread expression of the K^b molecule throughout the liver may lead to deletion of CD8 cells from repeated stimulation, though a previous study by our group has shown expansion of K^b-specific T cells in this situation which are rendered ‘functionally silent’ as demonstrated by impaired IFN-γ production in response to K^b stimulation [523]. To dissect the mechanisms by which tolerance is induced, it is helpful to consider how K^b molecules expressed in the recipient liver may be

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**Figure 51: Activation of K^b-specific T cells of varying affinity mediate destruction in naïve mice**

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recognised by the immune system. Possibilities include direct recognition by alloreactive CD8 T cells, presentation of K\textsuperscript{b} peptides by recipient K-haplotype class I molecules on hepatocytes, cross-presentation of K\textsuperscript{b} peptides on recipient K-haplotype class I molecules on APCs or presentation of K\textsuperscript{b} peptides by recipient APCs on MHC class II to CD4 T cells. As only the first of these pathways is dependent on direct antigen recognition, strategies to imitate this interaction may provide valuable insight into the process of tolerance acquisition.

4.2 Role of Direct Antigen Recognition in Establishing Tolerance

To further investigate the immunological mechanisms underlying allograft acceptance after rAAV Kb administration, we examined the role of direct antigen recognition. A mutant vector (rAAVD227K) was constructed with a point mutation from aspartic acid to lysine at residue 227 in the acidic loop of the K\textsuperscript{b} \( \alpha_3 \) domain, abrogating CD8 coreceptor binding and therefore direct antigen recognition by the majority of cytotoxic T cells. Administration of rAAVD227K (5 \( \times \) 10\textsuperscript{10} to 5 \( \times \) 10\textsuperscript{11} vgc) produced high-level, liver-specific expression of K\textsuperscript{b}-D227K on hepatocytes comparable to the expression of WT-K\textsuperscript{b} after rAAV Kb transduction. There was no evidence of hepatitis on serum ALTs or IHC staining. Allografts from 178.3 donors to B10.BR recipient mice one week after injection with 5 \( \times \) 10\textsuperscript{10} or 5 \( \times \) 10\textsuperscript{11} vgc rAAVK227K failed to achieve tolerance, with a median time to rejection of 27 days (range 16 – 33 days) in mice treated with 5 \( \times \) 10\textsuperscript{10} vgc rAAVD227K and 26 days (range 14 – 32 days) in mice treated with 5 \( \times \) 10\textsuperscript{11} vgc rAAVD227K. Rejection of the allografts followed a more indolent time course and different appearance macroscopically, suggesting that the cellular mechanisms
underlying rejection may be different than that seen in 178.3 allografts to naive B10.BR mice. More importantly, rejection demonstrates that direct antigen recognition by CD8-dependent clones has a fundamental role in establishing tolerance.

Several hypotheses to account for the delayed rejection following rAAVD227K administration are considered. Firstly, the small population of CD8 T cells possessing high affinity for K\(^b\) (which do not require CD8 co-receptor binding) and/or those recognising indirectly-presented K\(^b\) peptides are initially activated, but subsequently inactivated or deleted, whereas the bulk of alloreactive CD8 T cells remain naive.

When the graft displaying the WT-K\(^b\) antigen is applied 7 days after rAAVD227K inoculation, lower affinity CD8 dependent T cells populations are activated and proliferate, mediating rejection at a decreased tempo (see Figure 52).

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**Figure 52: Mechanism 1 - delayed rejection of allografts after K\(^b\)-D227K**
An alternative possibility is that CD8 independent T cells activated in the absence of competition from CD dependent cells expand more initially and can reject grafts applied with the first 1 – 2 weeks after transduction but would later be inactivated or deleted. A series of skin grafts has been performed in animals injected 3 weeks prior with rAAVD277K to determine whether tolerance then ensues (see Figure 53). This experiment is ongoing, but the first mouse grafted rejected skin with an identical tempo to mice transplanted 7 days after rAAVD227K inoculation, tending to support the first scenario. An additional possibility to account for the delayed rejection is that Tregs recognising processed K\(^b\) peptides on recipient APC dampen but do not completely prevent rejection by alloreactive CD8 T cells.

![Figure 53: Mechanism 2 - delayed rejection of allografts after K\(^b\)-D227K](image)

4.3 PD-L1 UPREGULATION ON HEPATOCYTES IS SEEN AFTER DIRECT ANTIGEN RECOGNITION OF DONOR MHC CLASS I IN RECIPIENT LIVER
Interactions between protein cell death ligand 1 and its receptor play an important role in the induction of tolerance. PD-1:PD-L1 interactions contribute to the functional suppression of reactive lymphocytes in the liver during viral infection, silencing antigen-specific CD8 responses through cross-talk between hepatocytes and cytotoxic T cells. PD-L1 is widely expressed within the liver and is upregulated in the context of IFN-γ secretion: its ligand PD-1 is found on T cells, B cells, NK cells and macrophages [362]. PD-L1:PD-1 interactions augment the production of regulatory T cells, induce apoptosis of reactive clones and inhibit TCR-dependent T cell proliferation [365, 370, 374]. Its prominent role in persistent hepatotropic viral infections is evidenced by PD-1 upregulation on exhausted virus-specific CD8 T cells and increased effector T cell function and subsequent viral clearance after PD-L1 blockade [375-380]. In hepatitis B, upregulation of PD-L1 on Kupffer cells and liver sinusoidal epithelial cells is associated with persistence of infection [598]. In particular, PD-L1 upregulation on hepatocytes in the context of viral infection interacts with PD-1 on T cells to induce apoptosis of reactive clones [387]. In a murine model of liver transplantation, blockade of PD-L1 in recipients abrogates apoptosis of infiltrating cells leading to acute rejection, indicating that PD-L1:PD-1 interactions are critical in the spontaneous acceptance of liver allografts [373].

FACS analysis of isolated hepatocytes revealed a rise in PD-L1 which peaks at around day 7 after transduction with rAAVKb. rAAVKb administration to C57BL/6 (b-haplotype) mice in which K^b is a self-antigen did not produce a rise in PD-L1, confirming that the increase in ligand expression is dependent on activation of recipient CD8 T cells by the transgene produce and is not a non-specific response to the vector itself. Widespread activation of K^b-sensitive lymphocyte populations may
generate enough IFN-γ to induce significant PD-L1 upregulation (as seen by FACS), subsequent ligation of PD-1 on CD8 T cells induces apoptosis and/or functional silencing leading to tolerance to K^b^ and long-term allograft acceptance. We have previously shown that K^b^-specific CD8 T cells are expanded in number after Kb-transduction but display diminished effector function with reduced IFN-γ secretion in response to K^b^ stimulation [523]. As PD-L1 is also implicated in generating effective regulatory T cell populations, multiple mechanisms are likely to be contributing to allograft acceptance including the generation of regulatory T cells able to suppress alloreactive CD8 T cells as well as cell-intrinsic defects induced by PD-1 engagement.

Conversely, animals inoculated with rAAVD227K failed to demonstrate PD-L1 upregulation on hepatocytes at days 2, 7 or 14. As only the small proportion of high affinity CD8-independent T cells can be directly activated after K^b^-D227K transduction, the subsequent low levels of IFN-γ secretion may not be sufficient to cause a detectable rise in PD-L1 expression. These activated CD8 T cells may ‘burn out’ with ongoing exposure to K^b^-derived peptides, or they may be silenced through PD-1:PD-L1 cross-talk with hepatocytes, or through additional co-inhibitory pathways such as those resulting from the interaction of galectin-9 with TIM-3.

4.4 INFLAMMATORY RESPONSE TO ANTI-PDL1 AND ISOTYPE CONTROL ANTIBODIES IN K^b^-TRANSDUCED ANIMALS

Mice receiving rAAVKb and anti-PDL1 concurrently (immediate blockade) showed macroscopic liver lesions and evidence of hepatitis with inflammatory cellular infiltrate
and raised transaminase levels. Lesions stained strongly for F4/80 revealing macrophages as the dominant infiltrating cell. Increased numbers of CD8 T cells were also noted. In contrast, mice receiving anti-PDL1 or rAAVKb alone demonstrated normal histology and ALTs. Importantly, the lack of an immune response after isolated anti-PDL1 administration shows that PD-L1 blockade does not lead to overt activation of self-reactive clones. Despite the immune response, strong ubiquitous expression of the K\(^b\) antigen on hepatocytes was maintained without significant evidence of hepatocyte destruction.

Interestingly, an inflammatory reaction was also seen in mice receiving rAAVKb and control antibodies. Initially, this was thought to be due to possible endotoxin contamination or reactivity of components of the polyclonal rat IgG with mouse hepatocytes and the antibody was changed from purified rat IgG to a functional grade rat IgG2ak antibody directed against keyhole limpet haemocyanin. This had little effect, with inflammatory infiltrates noted around the portal tracts along with scattered intra-lobular lesions similar to those noted with administration of rAAVKb with anti-PDL1. These lesions were dominated by a phagocytic infiltrate. There was a statistically significant rise in ALTs (mean 198 U/L, peak 290 U/L) but this was less pronounced than in those animals that received anti-PDL1 (mean 721 U/L, peak 844 U/L).

Anti-PDL1 administration in association with rAAVKb may allow the proliferation of K\(^b\)-reactive T cell clones normally silenced through PD-1:PD-L1 interactions. Examination of Kb-transduced livers under delayed blockade (anti-PD-L1 or isotype control antibodies administered 3 weeks after rAAVKb) was therefore performed when PD-L1
expression on hepatocytes had decayed and K\textsuperscript{b}-specific clones were presumably exhausted. As expected, mice receiving anti-PDL1 demonstrated a significant rise in ALTs in the immediate blockade group, whilst transaminase levels in the delayed blockade group were comparable to transduced mice receiving isotype control antibody. This suggested a degree of non-specific inflammation resulting from antibody administration to transduced mice induced by a generic mechanism unrelated to PD-L1 blockade. The more pronounced transient inflammatory reaction noted in those receiving rAAVKb and anti-PDL1 concurrently is overlaid on this reaction which appears to be persistent.

The non-specific inflammatory response may be a result of cellular activation mediated by the Fc portion of anti-PDL1 and isotype control antibodies. Fc-receptors are naturally expressed on NK-cells, Kupffer cells, macrophages and LSEC within the liver. The dominance of peri-portal and intra-lesion aggregations of F4/80\textsuperscript{+} cells could reflect activated Kupffer cells/macrophages effecting ADCC or damaging hepatocytes through the release of soluble mediators such as NO or TNF-\textalpha. Rodents also express the neonatal Fc receptor (FcRn) on hepatocytes [599]. FcRn is structurally related to MHC class I, being composed on an \(\alpha\)-chain and \(\beta\)-2-microglobulin that is vital for cell surface expression [600].

Administration of the rAAVKb vector and \(K^b\) overexpression may augment production of \(\beta\)-2-microglobulin, entraining the upregulation of FcRn receptors and potentiating an Fc-mediated response after antibody administration which in not seen in untransduced mice. If such an effect exists it would have implications in the clinical situation where human recipients often receive antibody therapy in the setting of
transplantation. Delayed blockade, whilst allowing tolerance to K\textsuperscript{b}-reactive clones and dampening the inflammatory reaction to immediate anti-PDL1 administration, may allow for increased FcRn upregulation after rAAVKb administration. Additionally, binding of Fc domains of administered antibodies to FcγRIII on NK cells promotes the production of pro-inflammatory cytokines such as IFN-γ which may stimulate K\textsuperscript{b}-reactive clones (not silenced due to PD-L1 blockade) and augment the immune response, explaining the higher ALTs seen in transduced animal treated with anti-PDL1 versus control antibody. Further experiments determining whether FcRn is upregulated in the setting of K\textsuperscript{b} overexpression, and replacing intact anti-PDL1 and control antibodies with Fab fragments will be needed to confirm whether this non-specific inflammation is an Fc-mediated effect but these experiments are beyond the scope of this thesis.

4.5 PD-L1 Blockade Fails to Break Tolerance in K\textsuperscript{a}-Transduced Animals

Administration of anti-PDL1 concurrent with rAAVKb failed to break tolerance, with ongoing graft survival at 91 days and no signs of rejection upon inspection of the graft, making it likely that these animals will demonstrate operational tolerance. Tolerance to 178.3 allografts in B10.BR rAAVKb-transduced animals as well as rejection after rAAVD227K inoculation is therefore not fully explained by differences in PD-L1 expression.

A number of explanations exist for the failure of anti-PDL1 to break tolerance. PD-L1 blockade theoretically allows the activation of reactive T cells, but due to the size of
the liver and widespread expression of Kb, these cells may subsequently ‘burn out’. This may mimic a situation of high-level antigen presentation with inadequate T cell help which is known to generate CD8 cells with an exhausted phenotype [601].

It is possible that there is upregulation of multiple ligands after rAAV Kb administration and that other receptor interactions are promoted and/or become dominant when PD-L1 is blocked. One such candidate is the galectin-9:TIM-3 pathway, which was originally recognised as a negative regulator of the Th1 response and promoter of peripheral tolerance [602]. Galectin-9:TIM-3 binding has been noted to suppress immune responses, thereby facilitating the persistence of hepatotropic viral infections by directing reactive TIM-3+ lymphocytes to functional exhaustion with an impaired ability to secrete IFN-γ and TNF-α as well as increasing their susceptibility apoptosis [586, 603, 604]. Whilst blockade of TIM-3 leads to T cell proliferation and secretion of effector cytokines, blockade of both PD-L1 and TIM-3 increase effector responses revealing that these pathway are non-redundant [586, 587]. Breaking tolerance may require blockade of multiple pathways such as PD-L1:PD-1 and galectin-9:TIM-3.

Furthermore, Fc receptors on resident hepatic cells may compete with the PD-L1 epitope for the anti-PDL1 antibody, leading to a false negative through incomplete blockade. To provide definitive evidence that PD-L1 blockade does not break tolerance, Fab fragments will need to be used in an attempt to eliminate competitive binding and the non-specific inflammatory response which may have contaminated results.
4.6 Evidence for iTregs in Controlling Cytotoxic T cell Responses

Administration of liver-targeted rAAV expressing various proteins has been shown to lead to the induction of transgene-specific regulatory T cells which suppress immune responses and can be adoptively transferred to confer tolerance [532, 534, 540]. We sought evidence for induction of Tregs which may be mediating suppression of K\textsuperscript{b}-specific lymphocyte clones.

Administration of rAAVKb to mice primed by rejecting a skin graft a week prior resulted in a mild increase in Treg populations concentrated around the portal tracts as seen by staining for FoxP3. It is interesting to note that regulatory T cells are also seen localised to portal tracts in chronic viral infections of the liver which are known to be associated with tolerance to the pathogen [605-607]. Treg expression was increased only in primed animals which subsequently received rAAVKb and not in primed uninjected controls. Cell counts in livers harvested 7 and 14 days after rAAVKb inoculation demonstrate mildly elevated CD8 T cell counts most pronounced at day 7 correlating with increased numbers of peri-portal FoxP3\textsuperscript{+} cells. There was also elevated B220 staining noted at the 7 day mark. As B220 is a marker for both B cells and apoptotic CD8 T cells, this may reflect Treg-mediated apoptosis of K\textsuperscript{b}-specific cytotoxic cells. Interestingly, this also correlates with the time of peak PD-L1 expression on hepatocytes, a ligand known to induce production of Tregs and apoptosis of reactive clones. These finding provide evidence that this iTreg population is dependent on hepatic K\textsuperscript{b}-transduction and furthermore suggest that these regulatory cells may have a role in suppressing the response of activated cytotoxic T cells. The presence of these Tregs may also contribute to limiting the rise in transaminases seen in this setting.
Further experiments incorporating ablation of FoxP3⁺ Tregs will be needed to confirm that these regulatory cells are restraining cytotoxic T cell responses.

Presentation of Kᵇ-derived peptides to CD4 T cells by recipient APCs may generate a population of Kᵇ-specific iTregs which suppress alloreactive CD8 responses. Moreover, PD-L1 is known to direct T cells towards a regulatory phenotype and FACS results show an appreciable increase in PD-L1 expression after rAAVKb administration. Another explanation for the induction of Tregs seen in primed animals receiving rAAVKb may be related to the recently discovered ‘Tregitope’ population, epitopes found within the constant Fc fragment of IgG (adjacent to the Fab hypervariable domains) and the highly conserved regions of Fab that can specifically activate nTregs [361]. ‘Tregitopes’ have been shown to reduce proliferation of CD8 T cells and effector cytokine secretion, expand nTreg pools, induce Treg formation from naïve T cell and convert effector T cells into adaptive Tregs, suppressing the immune response [360, 608]. It is thought that the Tregitope domains in IgG may explain the immunosuppressive effect long seen with intravenous immunoglobulin (IVIG). Interestingly, a fully mismatched murine skin transplant model demonstrated that IVIg bound to Tregs resulted in their functional activation and increased their capacity to suppress alloreactive responses thus conferring allograft protection [609]. The induction of FoxP3⁺ cells around portal tracts may therefore result from deposition of allo-IgG within the liver after Kᵇ-transduction in the primed host, thereby stimulating expansion of a regulatory T cell population through ‘Tregitope’ domains of IgG.

Kᵇ-staining revealed no apparent differences in expression on hepatocytes compared to transduced naïve mice, suggesting that immune-mediated destruction of
transduced hepatocytes in primed animals was not occurring. The liver tolerance effect presumably mediates tolerance of K\(^b\)-peptides even in the face of active immunity/memory responses which are considered a major barrier to transplantation. This has also been demonstrated in previous work by our group, where animals who rejected a 178.3 graft and were subsequently administered rAAVKb and re-grafted developed operational tolerance [523]. It is further supported by the ability of the liver to confer tolerance to peptides already proven to immunogenic, as demonstrated by the phenomenon of oral tolerance. Taken together, these observations suggest that the liver tolerance effect is a powerful arbiter of peripheral immunosuppression which can be harnessed to induce operational tolerance to allografts.
5.0 FUTURE WORK

To better delineate the process whereby K\(^b\)-D227K expression leads to delayed rejection, 178.3 allografts to B10.BR mice 3 weeks post-inoculation with rAAVD227K will be repeated. Tolerance to the grafts will suggest that high affinity CD-independent CD8 T cells are initially activated and able to destroy the graft in the first few weeks but are subsequently are burnt out leaving a population of ignorant cells, whilst rejection would provide evidence that low-affinity CD8-dependent T cells mediate destruction. Another avenue which needs to be explored is the non-specific inflammatory response observed when hepatocytes are transduced with K\(^b\) concurrently with antibodies (anti-PDL1 and isotype control). The resulting hepatitis may have contaminated the results of PD-L1 blockade which failed to break tolerance in this study. Work is currently underway to repeat these experiments with Fab fragments and thereby eliminate any Fc-mediated effects.

Similarities between the galectin-9:TIM-3 and PD-1:PD-L1 pathways in directing the immune response to tolerance make them attractive targets for investigation. Future FACS analysis will be extended to include markers for galectin-9 and TIM-3 proteins. If upregulation is detected the effects of galectin-9:TIM-3 blockade on skin graft survival will be examined to see whether it can break tolerance resulting from K\(^b\)-transduction.

Work is also underway to transfer this model from a B10.BR (k-haplo) recipient to a C57BL/6 (b-haplo) recipient in order to utilise the many genetic modifications available on the BL/6 background and further elucidate the mechanisms of tolerance induction. Preliminary data indicate that acceptance of B6 K\(^d\) skin grafts is prolonged in C57BL/6 mice expressing K\(^d\) on hepatocytes following inoculation with rAAVKd.
6.0 CONCLUSION

The development of liver-targeted hybrid recombinant adeno-associated viral vectors capable of inducing stable, high-level transgene expression in hepatocytes holds promise for harnessing the liver-tolerance effect to achieve operational tolerance to allografts. Importantly, there is mounting evidence that such tolerogenic effects are conferred even in the recipient with a pre-existing memory response. Such a response has been a significant barrier to achieving tolerance in human populations, which display high frequencies of alloreactive memory T cells pre-transplant.

Direct antigen recognition plays a pivotal role in conferring this tolerance, leading to upregulation of PD-L1 on \( K^b \)-transduced hepatocytes. Although complicated by an element of non-specific inflammation, antibody blockade of PD-L1 failed to break tolerance, suggesting that multiple pathways are involved in silencing or deletion of alloreactive CD8 T cells after liver-specific alloantigen expression in recipients. The recent demonstration that rAAV-mediated expression of \( K^d \) in the liver of b-haplotype CD57BL/6 recipients prolongs survival of \( K^d \)-bearing skin grafts is an encouraging sign that this tolerance induction strategy may be generalisable to other allogantigens and genetic backgrounds including the setting of clinical allotransplantation in human recipients.
7.0 REFERENCES


61. Shen, L., T.A. Potter, and K.P. Kane, *Glu227-->Lys substitution in the acidic loop of major histocompatibility complex class I alpha 3 domain distinguishes low avidity*


595. Bolacchi, F., et al., *Increased hepatitis C virus (HCV)-specific CD4+CD25+ regulatory T lymphocytes and reduced HCV-specific CD4+ T cell response in HCV-


	  

8.0	   APPENDIX:	  SEQUENCING	  H2-­‐KB	  AND	  SITE	  DIRECTED	  MUTAGENESIS	  
Legend:	  

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hAAT	  forward	  primer	  site	  	  
WPRE	  reverse	  primer	  site	  	  
Multiple	  cloning	  site	  
Kb	  coding	  region	  
hAAT-­‐Rev	  Primer	  site	  (marker)	  	  
AFor	  Primer	  site	  (marker)	  	  
ARev	  Primer	  site	  (marker)	  	  
WPRE-­‐For	  Primer	  site	  (marker)	  	  
	  

	  
Empty	  pAM	  vector	  
	  
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agcagcctcccccgttgcccctctggatccactgcttaaatacggacgaggacagggccctgtctcctca
gcttcaggcaccaccactgacctgggacagtgaatgcggccgctctagaactagtggatcccccgggctg
caggaattcgatatcaagcttatcgataatcaacctctggattacaaaatttgtgaaagattgactggta
ttcttaactatgttgctccttttacgctatgtggatacgctgctttaatgcctttgtatcatgctattgc
ttcccgtatggctttcattttctcctccttgtataaatcctggttgctgtctctttatgaggagttgtgg
cccgttgtcaggcaacgtggcgtggtgtgcactgtgtttgctgacgcaac

	  
	  
Sequencing	  empty	  pAM	  Vector	  (overlap	  shown	  in	  italics)	  
CAGCCTCCCCCGTTGCCCCTCTGGATCCACTGCTTAAATACGGACGAGGACAGGGCCCTGTCTCCTCAGC
TTCAGGCACCACCACTGACCTGGGACAGTGAATGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCA
GGAATTCGCCACCATGGTACCGTGCACGCTGCTCCTGCTGTTGGCGGCCGCCCTGGCTCCGACTCAGACC
CGCGCGGGCCCACACTCGCTGAGGTATTTCGTCACCGCCGTGTCCCGGCCCGGCCTCGGGGAGCCCCGGT
ACATGGAAGTCGGCTACGTGGACGACACGGAGTTCGTGCGCTTCGACAGCGACGCGGAGAATCCGAGATA
TGAGCCGCGGGCGCGGTGGATGGAGCAGGAGGGGCCCGAGTATTGGGAGCGGGAGACACAGAAAGCCAAG
GGCAATGAGCAGAGTTTCCGAGTGGACCTGAGGACCCTGCTCGGCTACTACAACCAGAGCAAGGGCGGCT
CTCACACTATTCAGGTGATCTCTGGCTGTGAAGTGGGGTCCGACGGGCGACTCCTCCGCGGGTACCAGCA
GTACGCCTACGACGGCTGCGATTACATCGCCCTGAACGAAGACCTGAAAACGTGGACGGCGGCGGACATG
GCGGCGCTGATCACCAAACACAAGTGGGAGCAGGCTGGTGAAGCAGAGAGACTCAGGGCCTACCTGGAGG
GCACGTGCGTGGAGTGGCTCCGCAGATACCTGAAGAACGGGAACGCGACGCTGCTGCGCACAGATTCCCC
AAAGGCCCATGTGACCCATCACAGCAGACCTGAAGATAAAGTCACCCTGAGGTGCTGGGCCCTGGGCTTC
TACCCTGCTGACATCACCCTGACCTGGCAGTTGAATGGGGAGGAGCTGATCCAGGACATGGAGCTTGTGG
AGACCAGGCCTGCAGGGGATGGAACCTTCCAGAAGTGGGCATCTGTGGTGGTGCCTCTTGGGAAGGAGCA
GTATTACACATGCCATGTGTACCATCAGGGGCTGCCTGAGCCCCTCACCCTGAGATGGGAGCCTCCTCCA
TCCACTGTCTCCAACATGGCGACCGTTGCTGTTCTGGTTGTCCTTGGAGCTGCAATAGTCACTGGAGCTG
TGGTGGCTTTTGTGATGAAGATGAGAAGGAGAAACACAGGTGGAAAAGGAGGGGACTATGCTCTGGCTCC
AGGCTCCCAGACCTCTGATCTGTC
CAGTGAATGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCGCCACCATGGTACCGTGC
ACGCTGCTCCTGCTGTTGGCGGCCGCCCTGGCTCCGACTCAGACCCGCGCGGGCCCACACTCGCTGAGGT
ATTTCGTCACCGCCGTGTCCCGGCCCGGCCTCGGGGAGCCCCGGTACATGGAAGTCGGCTACGTGGACGA
CACGGAGTTCGTGCGCTTCGACAGCGACGCGGAGAATCCGAGATATGAGCCGCGGGCGCGGTGGATGGAG

	  

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Sequencing: pAM2Akb (K^b sequence highlighted and position 227 identified)

Position 227: changed aspartic acid (codon GAC, single-letter data-base code D) to lysine (codon AAC, single-letter data-base code K)
TCGCTGTGGGCACTGACAATTCCGTTGGCTGCTGCC
TGTTGCCACCTGGAATTCTGCGGGGAGCTCCTCTGCTACTCCCTTGCCCTGAGCGGACC
TTCTCCTCCGGCCGCTCGCTGCGGGCTCTGCGGGCTCTTCCGCTCTGCGCTTTCCGGCCCTCAGACGAGTCC
GATCTCCTCCTTGGGCGGCTCCCGCATCGATACCTGCACTCGCTGATCGACTCCCTCAGCTGTCCTACTA
GTTGCCAGCCATCTGTTGTTTGCCTCCCTCCGGCTCTTGGACCCTGTGGGTGGTACCTCCACCTCGT
CTTCTCATATATAATGAGGAAATTGCAATCGCATTTGAGTTAGGTGTCATTCTATTTCTGGGGGTGGG
GTTGGCGCAGCAAGCAAGGGGGAGATGGGAAGACAATACGAGCTGCTGGGGATGCCAGGCTCTA
TGCTCTCAGGGCAGAGACAGCGGGGGGAGTTGGAAGCAATACGAGCTGCTGGGGATGCCAGGCTCTA
GGTTAATCATTAACAGAAAGCAAGAAGCTATGACTGATGGGACCTGGCACTCCCTCTCGCCCTCGCT
CACTGAGCCGGGAGACAAAGCCGCTGCGCCGAGGCCCAGGCTTCTGCCCCGGGGCCCTCAGTGAGCAGGCA
GGCCAGAGCCTTTTTGCAAAAGCCTAGGCCCTCCACAAAAAGCTCCCTCTACTACCTTCTGGAATAGCTAGA
GGCCGAGGCGGCTCCGCTCGCTGATAAATAAAATAAAATAATGACTCGCCATGGGGCGGAGATTGCGCGAA
CTGGCCCAATTTAGGGGGGATGGCGGAGTTAGGGGGGGGGAATAGCTGCTGACTATGAGATGCA
TGCTTTCGATACCTCTGCTGGGAGGCTG